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STIMULATION OF ACTIVE SODIUM TRANSPORT ACROSS THE ISOLATED TOAD BLADDER AFTER INJECTION OF ALDOSTERONE TO THE ANIMAL[1,2]

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ABSTRACT

Active sodium transport by the isolated urinary bladder of *Bufo marinus* was studied by means of the short-circuit current technique of Ussing and Zerahn. Sodium transport by this preparation was found to be greater when toads had been maintained in distilled water rather than in saline. This increase was associated with a higher concentration of circulating aldosterone. This hormone was shown to be produced by toad adrenal tissue.

Injection of aldosterone d, l 21-monoacetate to the animals the day prior to sacrifice resulted in a stimulation of sodium transport by the isolated bladder, whether the toads had been kept in saline or in distilled water. But after administration of aldosterone for 5 days, the activity of sodium transport was the same, regardless of environment.

The effect of habitat or aldosterone injections on active sodium transport by the isolated toad bladder did not influence the responsiveness of the preparation to the stimulation of sodium transport by vasopressin.

Bladder tissue water, sodium, potassium and chloride concentrations did not vary significantly despite large changes in sodium transport.

Administration of aldosterone to toads produced changes in plasma potassium, urine sodium and potassium concentrations opposite to those usually seen in mammals.

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ADMINISTRATION of aldosterone results in a decreased excretion of sodium in urine, through an enhanced reabsorption of this ion by renal tubule cells. As sodium reabsorption represents an active process (1, 2), it is reasonable to assume that aldosterone stimulates the mechanism responsible for the active transport of sodium from the tubular lumen to the interstitial fluid. A means of examining this hypothesis directly is provided by the toad bladder. This biological membrane, consisting of a single layer of mucosal cells supported by a small amount of connective tissue in which are scattered isolated bundles of smooth muscle, is capable of transporting sodium actively from its mucosal to its serosal surface (4); in so doing, it reabsorbs sodium from urine and is therefore comparable to the renal tubule.

Ussing and Zerahn (3) have established that net sodium transport by the frog skin is expressed quantitatively by the current required to nullify the potential difference spontaneously produced by this membrane. Leaf, *et al.* (4) have shown that this convenient relationship applies to the toad bladder. Hence, in the experiments to be reported, short-circuit current measurements serve as an index of the rate of sodium transport.

First, studies were undertaken to determine whether aldosterone is involved in the homeostasis of the toads used in these experiments (*Bufo marinus*). Then, the effects of aldosterone on sodium transport by the isolated bladder were studied following injection of the steroid into the animals before sacrifice.

MATERIAL AND METHODS

Experiments were performed during the Fall on toads *Bufo marinus* of both sexes partially immersed in groups of 2 to 3 in 1 L. distilled water or saline (125 mEq Na/L.) for 3 to 5 days before sacrifice. The animals were pithed, blood was obtained from the heart with a heparinized syringe and urine was collected from the bladder. A sample of the medium in which the toads were immersed was also saved. Both half-bladders of each toad were dissected and mounted successively[4] between lucite chambers (20 mm. diameter) as proposed by Ussing and Zerahn (3), with both surfaces bathed in 10 cc. aerated frog Ringer solution (Na: 113.5 mEq/L.; K: 1.88 mEq/L.; HCO_3: 2.39 mEq/L.; Ca: 0.89 mM/L.; osmolality: 220 mOsm/L.; pH: 8.1 at room temperature). Short-circuit current was measured at 5 minute intervals for the initial 30 minutes. One unit vasopressin (Pitressin, Parke, Davis and Co.) was then added to the serosal side, and the short-circuit current was determined, at 2.5 minute intervals for an additional 15 minutes. The bladders were then blotted, weighed immediately and again after 24 hours at 105° C, and prepared for sodium, potassium and chloride measurements by elution with a known volume of 0.05N nitric acid for at least 48 hours. Sodium and potassium concentrations were determined by flame photometry (Model KY-1, Baird Atomic, Inc.); chloride concentration, by the potentiometric method of Sanderson (5); osmolality, with the Fiske osmometer.

[4] The first half-bladder was studied within minutes after sacrifice, and the second half-bladder was used approximately one hour later. This delay resulted in an increased baseline short-circuit current and decreased response to vasopressin, whether the membrane was left in situ or placed in aerated frog Ringer solution during the interval. The electrical resistance of the membrane was not influenced by the delay.

Shortly after sacrifice, adrenals were excised as free of renal tissue as possible and stored frozen for incubation studies. Pools of adrenal glands from 25 to 30 toads were homogenized in ice-cold sucrose solution with a Potter-Elvehjem homogenizer prior to incubation for 3 hours at 37° C under a stream of 97.5% O_2—2.5% CO_2 in a Dubnoff incubator. The final composition of the incubation medium, modified from Eichhorn and Hechter (6) and Schönbaum *et al.* (7), was as follows (per L.): 200 mM sucrose, 5 mM niacinamide, 5 mM sodium fumarate, 5 mM $MgSO_4$, 1 mM ATP, 0.5 mM DPN, 0.5 mM TPN, 6.2 mM K_2HPO_4, 1.2 mM NaH_2PO_4, 10 mM dextrose, 10^7 units penicillin G potassium, 1 gm. streptomycin sulfate, 1 μc (18 μg) 4-C^{14} progesterone. After completion of incubation, the contents of the flasks were extracted three times with ethyl acetate, v/v, then the latter was evaporated under reduced pressure at 50° C. The dried residue was taken up for partition between water and petroleum ether, v/v, and the aqueous phase was extracted twice with methylene chloride which was then evaporated to dryness. The residue, dissolved in methanol, was applied to No. 1 Whatman paper for chromatographic development by the formamide-chloroform system of Zaffaroni (8) with appropriate steroid standards. The strips containing aldosterone and corticosterone were eluted separately with methanol which was then evaporated to dryness prior to chromatography in the system "B4" of Bush (9). The fractions isolated after this second step of purification were submitted to acetylation before chromatography in the system "B2" of Bush (9). The final eluates were dried and the residues dissolved for liquid scintillation counting (Packard Tricarb Spectrometer) in the following solution: 50 gm. naphthalene, 7 gm. diphenyloxazole, 50 mg. *p*-bis 2(5phenyloxazolyl) benzene, in 1 L. *p*-dioxane.

Aldosterone was injected into the toads as d, l 21-monoacetate. One mg. dissolved in 1 cc. ethanol, was diluted to 5 cc. with frog Ringer solution; 20 μg were injected subcutaneously into each animal daily, with the last dose administered 4 hours before sacrifice. When the excipient was injected to groups of control toads in the same amounts and on the same schedule, no effect was observed on the variables studied.

RESULTS

It was anticipated that toads placed in distilled water would lose enough sodium to the environment for stimulation of their sodium-conserving mechanisms to take place. Therefore, a larger secretion of aldosterone by the adrenal glands of these toads was expected with, as a result, an increase in sodium transport across their bladders. As shown in Table 1, the short-circuit current was indeed greater under such circumstances than when toads were kept in saline.

The concentration of circulating aldosterone was measured in 2 pools of approximately 30 cc. toad plasma[5] with the double isotope dilution derivation technique of Kliman and Peterson (10). Plasma collected from toads maintained in distilled water contained 1.17 μg of aldosterone per 100 cc., contrasting with a concentration of 0.68 μg per 100 cc. when toads were kept in saline. In the same plasma pools the concentrations of corticosterone were 2.64 μg per 100 cc. and 3.42 μg per 100 cc. respectively. Furthermore, it was demonstrated that aldosterone and corticosterone originate from toad adrenal cortical tissue as appears from incubation of

[5] As the volume of plasma generally did not exceed 2 cc. per toad, these pools represent blood collected from 15 animals at least.

TABLE 1. SHORT-CIRCUIT CURRENT AND ELECTRICAL RESISTANCE ACROSS BLADDERS FROM TOADS MAINTAINED IN DISTILLED WATER OR SALINE, BEFORE AND AFTER INJECTION OF ALDOSTERONE (means ±S.E.M.)

		Animals kept in distilled water			Animals kept in saline			Statistical significance of the differences*					
		Control	Aldosterone Treatment		Control	Aldosterone Treatment							
		(1)	1 day (2)	5 days (3)	(4)	1 day (5)	5 days (6)	(1)–(4)	(1)–(2)	(4)–(5)	(2)–(5)	(2)–(3)	(5)–(6)
Short-circuit current† μAmp./3.14 cm.²	Before Vasopressin	51.2± 3.0	78.4± 4.3	83.1± 6.3	30.0± 1.9	52.0± 3.0	85.2± 5.6	<0.001	<0.001	<0.001	<0.001	>0.5	<0.001
	After Vasopressin	64.4± 2.7	85.7± 3.7		49.0± 2.0	63.5± 2.7		<0.001	<0.001	<0.001	<0.001		
Electrical resistance‡ Ohms/3.14 cm.²	Before Vasopressin	304.5±35.1	404.4±26.5	296.1±32.2	329.3±29.8	433.6±32.0	219.8±23.1	>0.5	<0.05	<0.05	>0.4	<0.02	<0.001
	After Vasopressin	331.2±35.5	425.2±26.6		343.9±27.5	451.7±30.2		>0.7	<0.05	<0.02	>0.5		

* Using the "t" test of Fisher.
† Each value is the mean of 96 measurements.
‡ Each value is the mean of 32 measurements.

TABLE 2. PRODUCTION OF ALDOSTERONE AND CORTICOSTERONE BY TOAD ADRENAL HOMOGENATES

	Aldosterone (counts per minute)	Corticosterone (counts per minute)
Adrenal pool from toads kept in:		
distilled water	6.283	2.702
saline	7.666	3.468

1 μc 4-C^{14} progesterone was used as a precursor for each pool—efficiency of the counter, 40% approximately.

No correction was made for losses due to manipulations. As appears from subsequent studies, the recovery did not exceed 50% in all likelihood.

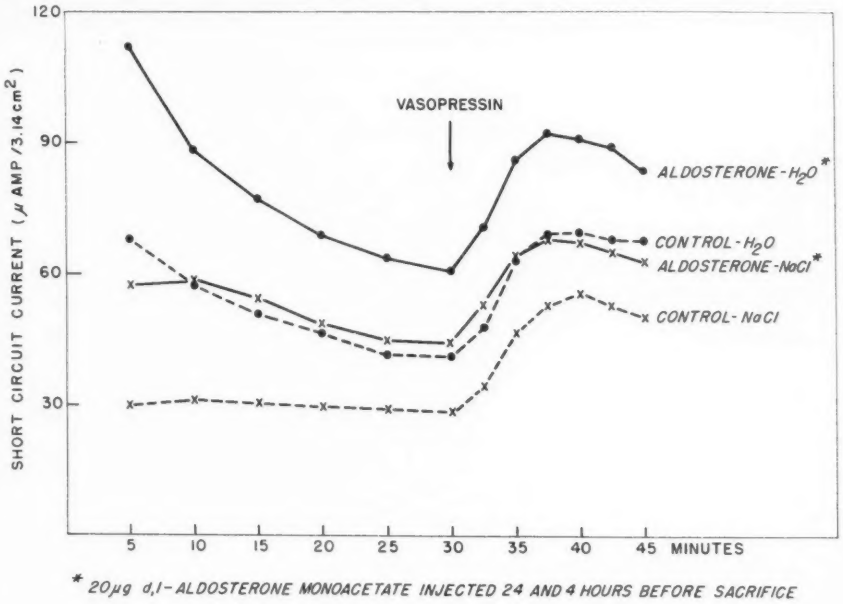

FIG. 1. Stimulation of active Na transport across isolated toad bladder by aldosterone *in vivo*

adrenal homogenates with C^{14} labelled progesterone, and recovery of radioactivity in the aldosterone and corticosterone fractions (Table 2).

After these observations lending support to the role of aldosterone in the stimulation of active sodium transport across the toad bladder, aldosterone monoacetate was injected to 2 groups of 8 toads the day prior to the experiments. The average short-circuit current recorded across the bladders from treated toads kept in saline approximated the values obtained with bladders from untreated toads maintained in distilled water (Table 1). But a further increase was observed in the latter group after injection of the hormone. The increase in short-circuit current across bladders from aldosterone treated toads averaged 53% when toads had been kept in distilled water, and 73% when they had been kept in saline; in absolute terms however, the hormonal effect was more pronounced with toads kept in distilled water.

Vasopressin, added after 30 minutes to the serosal side of the membrane, brought about a brisk increase in short-circuit current, as illustrated by Figure 1. The extent of the stimulation was independent of the pre-existing activity of sodium transport, being of about the same magnitude in all 4 groups.

The difference in short-circuit current across the bladder from toads kept

TABLE 3. WATER, SODIUM, POTASSIUM AND CHLORIDE CONCENTRATIONS IN TOAD BLADDER TISSUE
(means ±S.E.M.)

	Animals kept in distilled water			Animals kept in saline		
	Control****	Aldosterone treatment		Control****	Aldosterone treatment	
		1 day****	5 days		1 day****	5 days
Tissue water*	355.7	348.3	338.6	344.9	310.9	375.1
	±20.1	16.3	21.4	11.0	10.9	15.8
Tissue sodium**	458.4	546.0	496.7	515.0	507.3	535.3
	±18.7	34.4	29.2	27.4	16.8	23.1
Tissue potassium**	221.8	308.1	269.3	232.9	237.7	300.6
	± 6.7	12.9	15.2	8.3	9.3	12.2
Tissue chloride**	320.6	413.2	395.1	323.9	304.1	389.6
	±19.0	27.3	20.8	20.0	13.0	19.6
Tissue sodium***	137.6	159.0	145.7	151.9	154.9	146.0
Tissue water	± 6.6	9.3	10.2	10.1	6.1	8.4
Tissue potassium***	67.0	89.7	79.2	68.3	72.8	81.5
Tissue water	± 3.5	3.2	7.2	1.7	2.5	3.5
Tissue chloride***	94.4	119.7	116.5	93.3	94.5	106.4
Tissue water	± 3.7	6.9	9.8	6.3	2.8	7.2

* cc./gm. dry weight.
** μeq./gm. dry weight.
*** μeq./cc. tissue water.
**** Vasopressin added to the serosal surface 15 minutes before completion of experiment.

in saline or distilled water, persisted despite aldosterone administration for 1 day. Two additional groups of 8 toads were therefore given aldosterone daily for 5 days, in an attempt to eliminate by a more prolonged hormonal treatment the influence of habitat. Table 1 shows that this attempt proved successful since the mean short-circuit current measurements became identical, whether the toads had been maintained in distilled water or saline. Only the average short-circuit current across the bladder of treated toads immersed in saline showed a significant further increase. No vasopressin was used in these experiments.

Unlike the short-circuit current, the electrical resistance of the membranes to the passage of current[6] was not modified by the environment (Table 1). Aldosterone treatment produced an increase after 1 day, but a decrease after 5 days, of the resistance. No correlation could be drawn between the water and electrolyte content of the bladder tissue (Table 3) and the activity of sodium transport, or the electrical resistance, across this membrane.

Information derived from measurements of toad blood and urine is summarized in Table 4. The environment quite understandably induced large changes in blood and urine osmolalities and sodium and chloride concentrations. It also had an influence on the excretion of potassium, as evidenced

[6] The electrical resistance of the toad bladder was measured by adding 100 μAmp. to the short-circuit current and recording the resulting potential difference. It was determined at 15, 30, 37.5 and 45 minutes.

TABLE 4. COMPOSITION OF TOAD PLASMA, URINE AND IMMERSION "MEDIUM" (means ±S.E.M.)

Animals kept in distilled water					Animals kept in saline			
Control		Aldosterone treatment			Control		Aldosterone treatment	
3 days	10 days	1 day	5 days		3 days	10 days	1 day	5 days
				Plasma				
122.7 ±4.3	115.6 ±3.1	111.8 ±3.1	120.1 ±5.4	Sodium (m.eq./L.)	136.7 ±2.3	128.3 ±3.4	145.9 ±4.5	147.8 ±3.1
3.65 ±0.12	3.60 ±0.14	3.82 ±0.20	4.11 ±0.11	Potassium (m.eq./L.)	3.63 ±0.32	4.23 ±0.12	3.66 ±0.23	4.63 ±0.23
71.5 ±1.6	67.0 ±2.3	66.2 ±1.9	60.3 ±1.0	Chloride (m.eq./L.)	93.3 ±4.4	91.0 ±1.0	93.4 ±2.3	98.3 ±2.3
[248]	239 ±3.0	251 ±1.2	237 ±7.4	Osmolality (mOsm./Kg.)	[321]	295 ±2.0	[324]	337 ±4.4
30.6*	33.9 ±1.5	30.4 ±3.1	—	Blood hematocrit (%)	21.3*	28.5 ±2.0	23.7*	—
				Urine				
0.86 ±0.3	0.87 ±0.29	0.38 ±0.08	1.47 ±0.46	Sodium (m.eq./L.)	44.8 ±10.6	46.3 ±7.7	71.5 ±9.2	100.4 ±8.9
0.87 ±0.22	0.85 ±0.25	0.38 ±0.14	0.51 ±0.18	Potassium (m.eq./L.)	4.16 ±1.6	4.29 ±1.87	3.25 ±0.74	0.57 ±0.18
0.55 ±0.25	—	0.10 ±0.03	—	Chloride (m.eq./L.)	42.9 ±7.5	36.5 ±9.8	61.4 ±10.0	106.6 ±10.2
[57]	35.0 ±1.7	44.0 ±1.5	38.5 ±2.4	Osmolality (mOsm./Kg.)	[243]	185 ±21.2	[263]	303.5 ±17.1
				"Medium"				
0.66	0.53	1.00	0.65	Sodium (m.eq./L.)	—	—	—	—
0.48	0.24	0.24	0.20	Potassium (m.eq./L.)	0.75	1.09	1.14	1.15
0.39	0.06	0.75	0.37	Chloride (m.eq./L.)	—	—	—	—
43	20.5	26.5	22	Osmolality (mOsm./Kg.)	302	277	298	314

Values in brackets were obtained on pools. Values for "medium" and those with an asterisk represent the means of 3 or 4 samples each; therefore, no S.E.M. is included for them.

by the higher concentration of this ion in urine and in the "medium" when animals were kept in saline.

After aldosterone administration, the urine sodium and chloride concentrations were strikingly increased when toads were kept in saline. As for potassium, its concentration increased in blood and decreased in urine, under hormonal treatment. The results obtained for toads given aldosterone for 5 days were not due to a more prolonged stay in distilled water or saline as appears from data on untreated toads maintained in such environments for 10 days.

DISCUSSION

It has thus been demonstrated that aldosterone is a substance normally secreted by the adrenal tissue of the toad *Bufo marinus*, and that it is present in the blood of this species in sizable concentrations dependent, to a certain extent at least, on the habitat.

More than 20 years ago, Krogh (11) reported that frogs, after a month spent in distilled water, were able to "pump in" chloride to the extent that this ion could no longer be measured in tap water into which the animals

had been transferred. After placing frogs in distilled water for a few days, an increase in short-circuit current across the isolated frog skin was reported by Jørgensen (12). Maetz *et al.* (13) made the same observation and assumed that aldosterone secretion was stimulated under such circumstances. They considered this stimulation as the cause for the increased short-circuit current. It is their work that prompted the use of a distilled water or saline habitat for the toads as a means to influence their endogenous secretion of aldosterone, and thereby active sodium transport across their bladders. This approach was preferred to adrenalectomy because, although the latter has been performed on amphibians (14, 15, 16), the anatomical relationship between the adrenal gland and the kidney is such, in *Bufo marinus* at least, that the renal function is bound to be markedly compromised after complete destruction or removal of the adrenal tissue.

The presence of aldosterone has been reported in lower vertebrates; it has been isolated from the blood of the spawing male sockeye salmon (17) and after incubation of interrenal tissue of the killifish (18). Carstersen *et al.* (19) have shown it to be quantitatively the major steroid recovered after incubation of adrenal slices from the bullfrog *Rana catesbiana*. It is apparent, from the present studies, that aldosterone represents an important fraction of the adrenal secretion of *Bufo marinus*. With radioactive progesterone as a precursor, twice as much activity appeared in the aldosterone fraction as in the corticosterone fraction.

It would have been interesting to compare the production of aldosterone by adrenal homogenates from groups presumably secreting the hormone at a different rate. Unfortunately, no reliable standards of reference for such a study were available. The weight of adrenal tissue could not be determined owing to contamination by adhering renal parenchyma. On the other hand, the production of corticosterone cannot be taken as an index of the amount of incubated adrenal tissue because the transformation of progesterone into corticosterone is probably not independent from the environment to which the toads were exposed. Eisenstein and Hartroft (20) have indeed shown, by incubation of rat adrenal slices, that the rate of production of corticosterone as well as that of aldosterone was modified by sodium depletion. In contrast with the results of adrenal homogenate incubations, the concentration of corticosterone in plasma was higher than that of aldosterone. This apparent discrepancy may be due to the shorter biological half-life of aldosterone, as has been observed in man (21, 22). It is noteworthy that the concentration of aldosterone in toad plasma is at least ten times that found in normal man (22, 23, 24).

The increased concentration of circulating aldosterone after exposure of toads to distilled water is thought to be the reason for the increase in net sodium transport demonstrated across the bladders of these animals because administration of exogenous aldosterone also had this effect. Maetz *et al.* (13) showed that injections of aldosterone for a week to frogs kept

in saline brought the short-circuit current readings to the values found when frogs had been kept in distilled water. With the toad bladder, one day of treatment with the hormone was sufficient to induce definite changes in short-circuit current. Also, injection of aldosterone to toads kept in distilled water increased the short-circuit current further. The persistence of a difference in sodium transport by bladders from toads immersed in saline or distilled water despite one day of administration of relatively large doses of aldosterone, could be interpreted as meaning that aldosterone was not the sole factor involved in the influence of habitat. However, after 5 days of treatment with steroid, sodium transport proceeded at the same rate across the bladders of both groups of toads. Studies carried out in man have already revealed that, even when large doses of aldosterone were used, more than one day of administration was required before a maximum degree of sodium retention by the kidney could be achieved (25, 26).

Williams and Angerer (27) have recently reported that the potential difference across the isolated frog skin was decreased by adrenalectomy and stimulated after injection of adrenal cortical extract to the animals. These changes are probably the consequence of changes in the activity of sodium transport by the preparation. It is apparent, from Table 1, that the potential difference across the toad bladder was increased by stimulation of endogenous aldosterone secretion or after administration of the hormone. An increased sodium uptake across the Axolotl skin has been observed after injections of ACTH to the animal (28); whether this effect can be accounted for entirely by a stimulation of aldosterone secretion is impossible to claim, however. The stimulation of sodium transport resulting from addition of vasopressin *in vitro* to the serosal surface of the toad bladder (4) was found to be merely superimposed upon the previous activity of this process. This is also in agreement with the results of Maetz *et al.* (13) with the isolated frog skin.

Despite a substantial stimulation of net sodium transport across the toad bladder, and presumably the toad skin and renal tubule, there was no conspicuous effect of aldosterone treatment on blood hematocrit or plasma sodium and chloride concentrations and osmolalities. One possible reason is that in toads kept in saline a rise in glomerular filtration rate nullified the results of a stimulation of sodium transport.

The effects of aldosterone on urine sodium concentration, and on the concentration of potassium in plasma and urine, are the opposite of what is observed in mammals given the hormone, for reasons that are not apparent.

The lack of significant changes in bladder tissue composition despite large variations in sodium transport induced by aldosterone and environment corresponds to similarly negative findings in isolated toad bladders exposed to vasopressin (Leaf, unpublished data).

These observations establish the physiological significance of aldosterone for the toad *Bufo marinus*, and they were considered essential preliminaries to an attempt, actually successful (29), to demonstrate a direct stimulation of active sodium transport by the isolated toad bladder preparation upon addition of aldosterone *in vitro*.

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THE EARLY PHASE OF THYROID HORMONE FORMATION: STUDIES IN TISSUE SLICES[1]

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ABSTRACT

Sheep thyroid slices were incubated for various periods of time in buffered solutions containing I^{131}. The nature and the distribution of labeled components were determined.

The slices accumulated up to 70% of flask I^{131} during 1 hour, but a large fraction of this iodide was not bound to protein as PBI^{131}.

Slices were homogenized and PBI^{131} content of the separated nuclear, mitochondrial, microsomal, and supernatant fractions of the homogenate was determined. No definite evidence of transfer of particulate PBI^{131} to the soluble fraction of the cell was obtained, although this phenomenon was suggested by some experiments. Labeled MIT and DIT were present in thyroglobulin within 2 minutes after start of incubation, but thyroxine and triiodothyronine were not identified. In most experiments DIT/MIT ratio increased gradually during incubation.

The only soluble labeled protein identified was thyroglobulin.

THE process of thyroid hormone biogenesis *in vivo* is presumed to proceed by way of the concentration and oxidation of iodide, binding of the oxidized iodine atom to tyrosine of thyroglobulin with production of monoiodotyrosine (MIT) and diiodotyrosine (DIT), and subsequent coupling of iodotyrosines to form iodothyronines. Thyroglobulin, according to some observers, is iodinated after its secretion into the colloid (1). Protein firmly attached to cell particulate elements (nuclei, mitochondria, and microsomes) is also iodinated. The formation of soluble iodoproteins other than thyroglobulin in normal and abnormal human and animal tissue has been observed (2, 3, 4). The latter proteins have not been purified but appear to be of smaller molecular size than thyroglobulin. Their significance in hormone synthesis is unknown. Metabolism of iodide in thyroid slices *in vitro* is apparently similar to the *in vivo* process, although evidence of formation of iodothyronines is usually lacking (5, 6).

Little information is available on the phase of hormonogenesis occurring during the first few minutes. It seemed possible that important precursor-product relationships could be observed during this period, while such relationships might be obscured at a more remote interval after I^{131} labeling

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of the gland by the accumulated mass of labeled thyroglobulin. The studies reported here were undertaken on sheep thyroid slices to determine if there is accumulation of organic iodine within a subcellular fraction before its entry into soluble thyroglobulin, if MIT appears prior to DIT, and if peptide precursors of thyroglobulin are formed. Parallel observations on hormonogenesis in rat thyroids studied *in vivo* are described in an accompanying paper (7).

METHODS

Sheep thyroid slices 0.5 mm. thick were prepared with a Stadie-Riggs tissue slicer. Washed slices weighing in total 250 mg. were incubated in 5 ml. of Krebs-Ringer phosphate buffer for the desired time interval at 37° in a mechanical shaker in 25 ml. flasks exposed to air. The buffer contained 1 millimicromole KI^{127} and 5–10 μc NaI^{131} per ml. After incubation slices were rinsed, blotted, cooled to below 4° C, and homogenized in .25 M sucrose within 1 minute. An aliquot of homogenate was fractionated by centrifugation into nuclear, mitochondrial, microsomal, and soluble (supernatant) fractions (8). The last fraction presumably includes soluble cell proteins and colloid. Total I^{131}, PBI^{131} (protein bound iodide^{131}), and protein content were determined on each fraction, as well as the distribution of I^{131} among the amino acids of the labeled proteins.

Protein hydrolysis was accomplished by incubation with pancreatin (0.1 mg. per mg. protein) in solutions buffered at pH 8.4, for 48 hours at 37° C (9). Samples were chromatographed with and without prior hydrolysis in the n-butyl alcohol-2N acetic acid (1:1, v/v) or n-butyl alcohol-ethanol-2N ammonium hydroxide (5:1:2, v/v/v) systems using the ascending technique. Free iodide was separated from PBI^{131} by chromatography prior to hydrolysis. Location of labeled compounds was achieved by the addition of carrier amino acids and stable KI as previously described (10).

I^{131} content of samples was determined in a well scintillation gamma ray detector. Paper electrophoresis was done as previously described (10). Starch gel electrophoresis was done according to the technique devised by Smithies (11), and location of labeled components was determined by serially counting portions of the gel in the gamma ray detector. Protein content of samples was determined by the method of Lowry (12).

Results from thirteen slice studies are included in this report. Numbers appearing in text and figures identify the study from which the data were taken.

RESULTS

I^{131} uptake and incorporation into protein

Slice uptake of I^{131} increased with time, reaching 17–70% of total I^{131} present in the incubating flask at 60 minutes (Fig. 1). Average uptake for all studies at 60 minutes was 53%. The uptake of different preparations varied widely, apparently due to differences in the functional state of the sheep tissue obtained. Slice/incubating fluid iodide^{131} ratios were not directly determined. However, an accumulation of 5% of total flask I^{131} as free slice iodide^{131}, which would represent a ratio of 1:1, was exceeded in each experiment by the end of 10 minutes' incubation.

In the early minutes of incubation much of the collected I^{131} was present as free iodide, not found to protein. This free iodide constituted over 84% of total slice I^{131} at 1 minute, 50–85% at 20 minutes, and 5–52% at 120

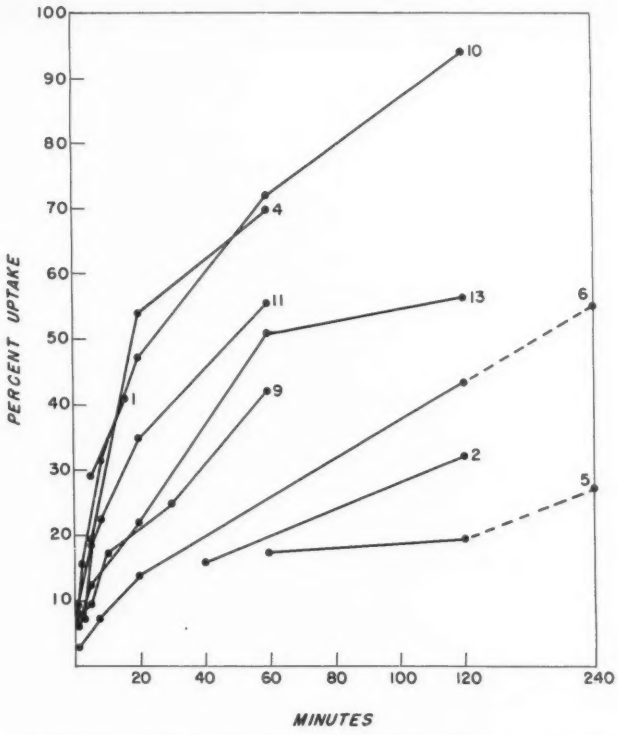


FIG. 1. Uptake of I^{131} by thyroid slices from incubation media during periods up to 240 minutes is depicted.

minutes (Fig. 2). Average free slice iodide at 60 minutes, for all studies in which these data were determined, was 49%. No correlation was observed between percent uptake at 60 minutes, and percent of slice iodide remaining free.

Distribution of slice PBI^{131} between sub-cellular components and supernatant fraction (Fig. 3)

In three experiments (6, 8, and 11) the proportion of total slice PBI^{131} present in the supernatant fraction was 40–83% at 1 minute, rose rapidly to 87–95% at twenty minutes, and then remained at this level during the subsequent period of observation. In three other experiments on which comparable data were obtained (4, 5, and 10), a similar increase in the proportion of total slice PBI^{131} present in the supernatant fraction was not observed. This difference in behavior did not correlate with either slow or rapid uptake and binding of I^{131} by the slices. The proportion of total slice PBI^{131} present in the combined particulate fractions (nuclei plus

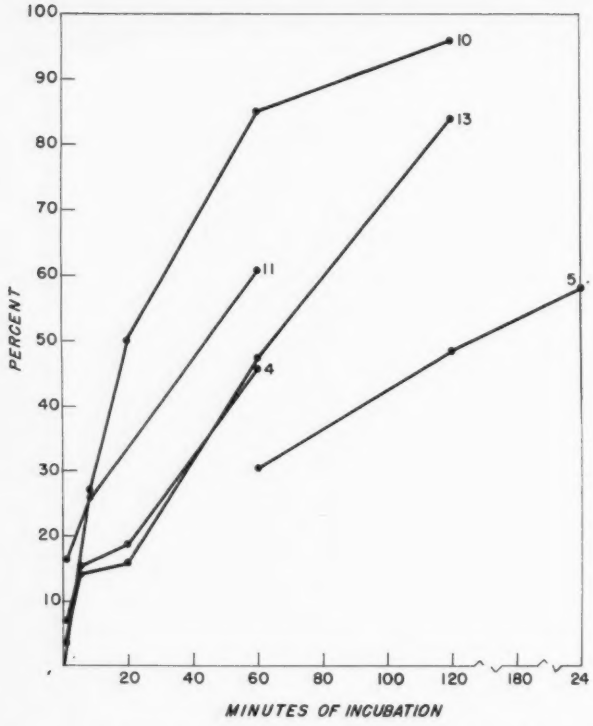
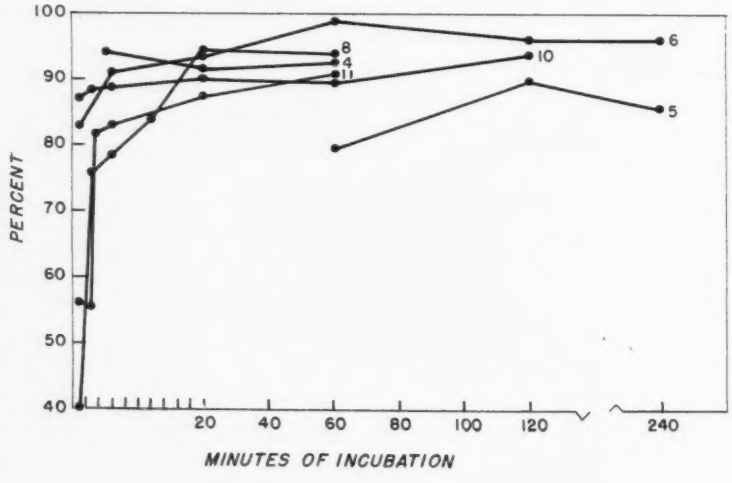


FIG. 2. The percent of total accumulated I^{131} which was bound to protein (PBI^{131}) at intervals during incubation is graphed. Note the small proportion of iodine which was bound during the early minutes of incubation.



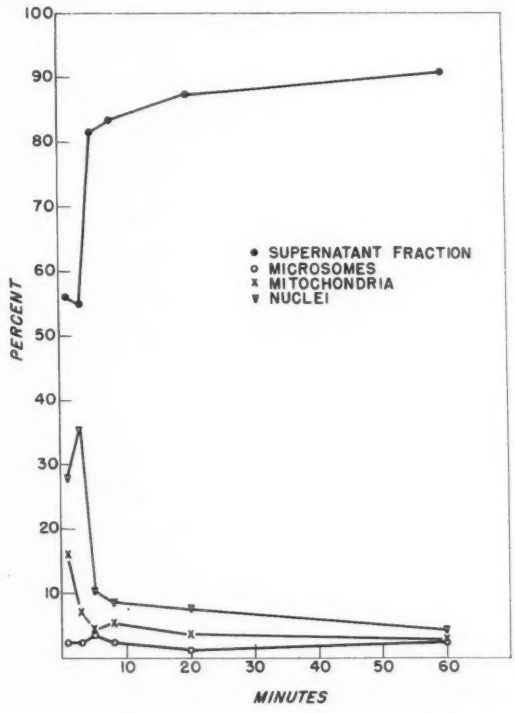


FIG. 4. The diagram details distribution of total slice PBI^{131} among the four centrifugally separated homogenate fractions prepared from slices. The increase during incubation in proportion of total slice PBI^{131} present in the supernatant fraction would be consistent with a transfer of PBI^{131} from particulate to soluble components of the tissue.

mitochondria plus microsomes) necessarily moved in an opposite direction to that in the supernatant fraction. The nuclear fraction usually contained more PBI^{131} at all times than the mitochondrial fraction, which in turn contained more than the microsomal fraction. It must be noted, however, that the nuclear fraction undoubtedly contained broken cells and contaminating mitochondria and microsomes, in addition to nuclei. The mitochondrial fraction likewise probably contained contaminating microsomes. Although the total PBI^{131} in each fraction increased at each time interval,

FIG. 3. In Figs. 3 and 4, percent of total slice PBI^{131} appears on the ordinate, and incubation time on the abscissa. The percent of total slice PBI^{131} present in supernatant fraction of homogenates prepared from incubated slices is shown. There was a progressive increase in the proportion of PBI^{131} present in supernatant fraction during incubation of slices of series 6, 8, and 11, but this was not observed in series 4, 5, and 10.

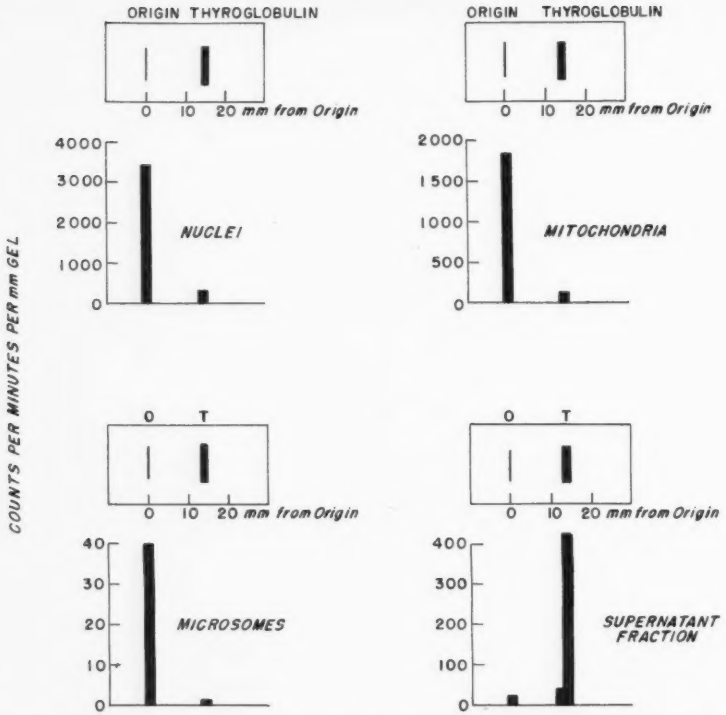


FIG. 5. Aliquots of nuclear, mitochondrial, microsomal, and supernatant fractions of the homogenate prepared from slices incubated for 60 minutes were subjected to starch gel electrophoresis. The anodal migration of protein components is shown on the abscissa, and PBI^{131} content on the ordinate. In the particulate fractions over 90% of PBI^{131} remained at the origin. In the supernatant fraction over 90% moved with thyroglobulin.

only the supernatant fraction progressively accumulated PBI^{131} relative to PBI^{131} in the other cell fractions. Distribution of total slice PBI^{131} in each fraction of the slice homogenate is diagrammed in Figure 4 for one study.

Particulate fraction PBI^{131} was not simply contaminating soluble thyroglobulin. This is evident from the diagram of starch gel electrophoresis of the particulate fractions showing that over 90% of this PBI^{131} remained at the origin, in contrast to the anodal migration of supernatant fraction PBI^{131} under similar conditions. Particulate fractions did contain about 10% of their PBI^{131} as soluble thyroglobulin (Fig. 5).

Distribution of I^{131} or PBI^{131} was not identical to that of protein. Eighteen to 29% of total slice protein was found in the nuclear fraction, 10–18% in the mitochondrial, 1–6% in the microsomal, and 52–70% in the supernatant fraction. There was no progressive alteration in this distribution during four hours' incubation.

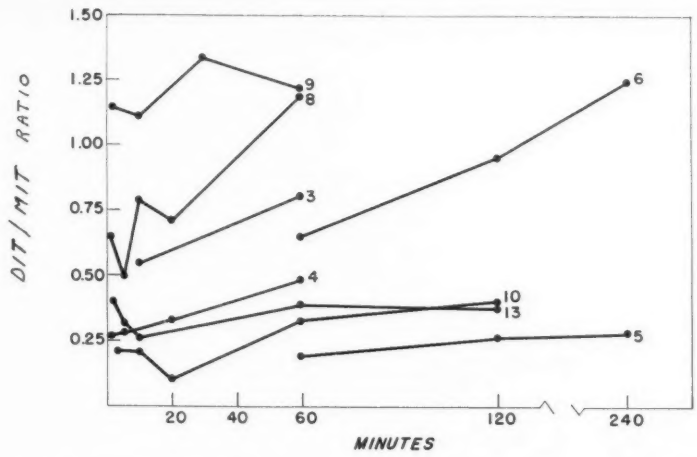


FIG. 6. The labeled DIT/MIT ratio present in digests of slice homogenate supernatant fractions appears on the ordinate, and the time of incubation on the abscissa. A distinct increase in this ratio was observed in series 3, 4, 6, 8, and 10.

Iodinated organic compounds formed

Pancreatin digestion of homogenate or subcellular fractions resulted in liberation of monoiodotyrosine and diiodotyrosine, but thyroxine and triiodothyronine were not definitely observed. The thyroxine and triiodothyronine carrier areas contained 0 to 7% of total radioactivity, but the proportion did not increase with time. On radioautography this I^{131} did not overlap the stable iodothyronine markers. This material was not studied in multiple solvent systems, but was assumed to be other than labeled iodothyronines. Usually less than 10% of PBI^{131} remained undigested after 48 hours.

The ratio of diiodotyrosine/monoiodotyrosine in homogenates prepared from slices ranged from 0.2 to 1.3. It was not related to percent uptake of I^{131} by the slice. Although the diiodotyrosine/monoiodotyrosine ratio tended to increase with time, the ratio was largely a function of the particular preparation being studied (Fig. 6). Thus, variations between slice preparations were greater than changes with time during incubation of any given preparation. Although data are insufficient for statistical analysis, groups which demonstrated an increasing DIT/MIT ratio during incubation (3, 4, 6, 8, 10) also had a higher than average uptake at one hour (56%) and less free iodide at the time (34%). Conversely, groups 5, 9, and 13, which did not have an increase in DIT/MIT ratio, had lower than average uptake (37%) and more free iodide (63%) at one hour. As early as iodinated tyrosines were detectable, both mono- and diiodotyrosine were always present (Table 1). The ratios of DIT/MIT were grossly similar in

TABLE 1. PANCREATIN DIGESTS WERE PREPARED FROM ALIQUOTS OF INCUBATING FLUID, WHOLE SLICE HOMOGENATE, AND HOMOGENATE FRACTIONS AFTER SLICES WERE INCUBATED FOR 2 TO 120 MINUTES

The percent of I^{131} in ascending chromatograms appearing at the origin and in the areas of stable DIT, MIT, I, T4 (thyroxine) and T3 (triiodothyronine) markers is tabulated.

	Time (mins.)	Origin	DIT	MIT	I	T4	T3
Incubating fluid	2	0.2	0.2	0.8	97.9	0.3	0.2
	5	0.2	0.2	0.7	97.1	1.5	0.2
	20	0.3	0.5	1.1	96.8	0.6	0.3
	60	0.6	0.7	2.5	92.0	3.4	0.4
	120	1.0	1.2	5.8	97.2	3.4	0.5
Homogenate	2	0.3	0.4	1.0	96.6	1.0	0.2
	5	1.4	2.0	6.0	88.0	1.1	0.4
	20	1.6	2.2	8.6	84.5	1.7	0.4
	60	5.7	8.5	21.9	59.1	2.0	0.5
	120	11.9	14.2	39.1	27.2	1.5	0.7
Nuclear fraction	2	2.6	4.2	15.4	70.8	1.8	1.7
	5	3.8	4.0	19.2	64.6	1.8	1.8
	20	4.2	4.0	22.4	60.0	3.7	1.2
	60	6.1	8.4	31.0	45.0	2.1	1.1
	120	11.6	14.6	40.0	23.8	1.9	0.8
Mitochondrial fraction	2	2.5	2.8	19.2	57.6	5.7	2.8
	5	4.0	2.4	23.3	53.8	3.4	5.6
	20	3.8	7.0	24.5	49.8	2.9	5.8
	60	7.1	6.0	33.9	43.9	2.6	2.1
	120	9.1	9.0	39.5	33.3	2.1	1.9
Microsomal fraction	2	2.7	4.8	16.5	58.3	7.6	4.9
	5	5.1	6.7	25.1	45.6	4.9	2.9
	20	5.8	6.1	19.6	52.0	2.7	4.3
	60	1.4	6.8	25.9	44.0	2.0	3.5
	120	8.4	9.4	31.2	37.1	1.9	2.3
Supernatant fraction	2	0.2	0.4	1.4	94.9	1.4	0.5
	5	1.2	1.8	4.9	90.0	1.1	0.5
	20	1.0	1.6	5.6	90.6	0.9	0.1
	60	5.6	7.7	20.0	62.5	2.1	0.1
	120	10.6	19.3	36.0	29.0	1.7	0.3

each homogenate fraction except the mitochondrial, in which fraction DIT/MIT ratios tended to be lower (Fig. 7).

Proteins iodinated

In starch gel electrophoresis studies of preparations incubated from 1 minute to 4 hours, the only iodinated soluble protein detected was thyroglobulin (Fig. 8). Insoluble protein attached to cell particles was also iodinated, and remained at the origin on electrophoresis, as shown in Figure 5.

DISCUSSION

Uptake of I^{131} by the thyroid slices increased progressively with time, and there was no marked decrease in rate during 4 hours of incubation. Accumulation of iodide in excess of binding was apparent, suggesting that iodide transport was not the rate limiting step in these preparations. This

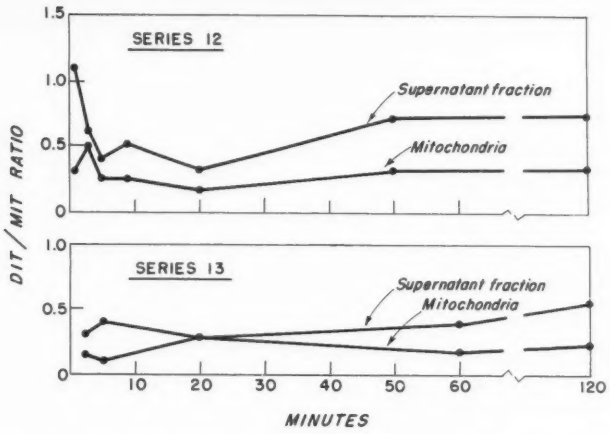


FIG. 7. The labeled DIT/MIT ratios in digests of proteins in mitochondrial and supernatant fraction are diagrammed at intervals during incubation. Supernatant fraction maintained a higher ratio.

finding is similar to the observations of Ingbar and Freinkel on rat thyroids *in vivo* (13).

The increase in proportion of total slice homogenate PBI^{131} present in the supernatant fraction and relative decrease in particulate fraction PBI^{131} observed during the first twenty minutes of incubation of three preparations is compatible with a process of transfer of particulate PBI^{131} to soluble fraction. An alternative explanation could be an early saturation of particulate protein iodinating sites, with continued iodination of soluble protein.

Identity of the insoluble particulate fraction PBI^{131} is not known. The particulate PBI^{131} could be (1) thyroglobulin firmly attached to cell particles, (2) a peptide precursor of thyroglobulin attached to cell particles, (3) unrelated iodinated particulate proteins. Either of the first two possibilities would fit with a hypothetical transfer of PBI^{131} from particulate to soluble fraction. The last possibility is suggested by the difference in iodinated amino acid analyses between particulate and soluble fraction protein. The DIT/MIT ratio was observed to be lower in mitochondrial than supernatant fraction. A similar discrepancy between particulate and supernatant fraction iodinated amino acid analysis has been reported by Robbins *et al.* (2). The particulate PBI^{131} of the nuclear fraction is thought to be in cytoplasmic particles attached to the nuclei, but not in the nuclei themselves (14).

Most available evidence on the site of iodination of thyroglobulin is derived from autoradiographic studies (1, 15, 16). The observations of Wollman (1), in particular, support the concept that iodination occurs on

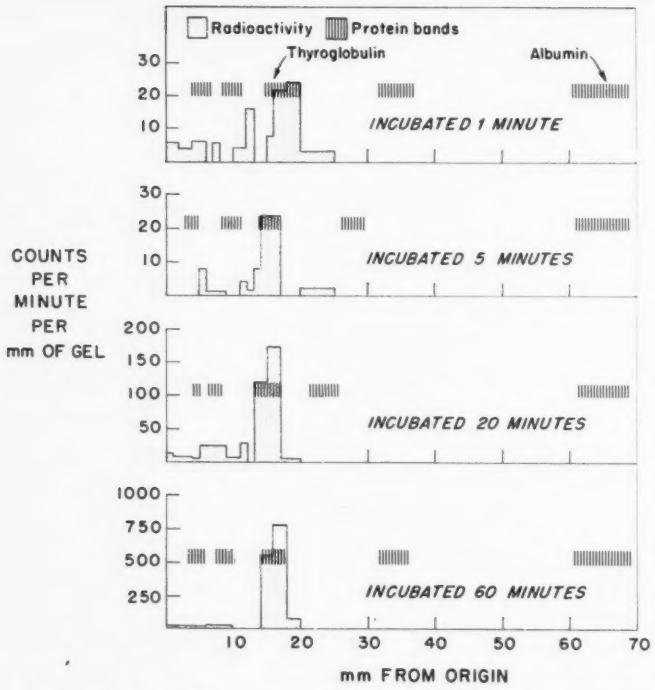


FIG. 8. Distribution of protein components and PBI^{131} are shown for supernatant fraction of slices after various periods of incubation. Anodal migration of proteins is depicted on the abscissa, and PBI^{131} content on the ordinate. Only the thyroglobulin band was consistently labeled.

pre-formed thyroglobulin inside the colloid. This hypothesis requires that the "oxidized" form of iodine diffuses from the cell into the colloid, or that trapped iodide is oxidized within the colloid. The latter concept is especially difficult to support, since no oxidizing system has been found in the soluble constituents of thyroid tissue. Surely some iodination of protein occurs within the cells, as evidenced by the appearance of cell particulate PBI^{131} in this and other studies (2, 4, 10), and the appearance of PBI^{131} in fetal thyroid glands prior to formation of colloid (17, 18). Whether the particulate PBI^{131} can indeed serve as precursor of soluble iodinated thyroglobulin, as suggested by the relative decrease of particulate PBI^{131} during incubation, was not established by this study. The possibility of transfer of particulate PBI^{131} to soluble fraction is being investigated further at present in this laboratory.

Soluble thyroglobulin was iodinated within 1 minute. This observation suggests that protein synthesis is completed within one minute, or that iodination takes place in previously formed but non-iodinated proteins.

The iodination is not simply an exchange reaction, as added stable iodide will not remove an I^{131} label from thyroid tissue *in vivo* or *in vitro* (5).

Both MIT and DIT were present in the earliest hydrolysates. The ratio of diiodotyrosine to monoiodotyrosine tended to increase with time, and a stable ratio had probably not been reached by 4 hours. The second iodination of tyrosyl followed so quickly after the primary iodination that monoiodotyrosine was not observed as the only iodinated constituent of the slice. That there was no radical change in the ratio of DIT/MIT in 4 hours of incubation suggests that monoiodotyrosine is not simply a precursor of diiodotyrosine. Perhaps a portion of monoiodotyrosine formed undergoes an immediate second iodination, while another portion is not further iodinated. This specificity of iodination might depend on steric arrangement in thyroglobulin, iodide supply, or other factors, and would not contradict the presently accepted hypothesis of a single enzyme controlling the formation of mono- and diiodotyrosine. An alternative to this concept would be the progressive total conversion of MIT to DIT. To maintain constant labeled DIT/MIT ratios at some period remote from the initial labeling, it would then be necessary to assume rapid degradation of labeled iodoamino acids and recycling of the liberated iodide; otherwise all labeled MIT would be converted to DIT. Available evidence does not permit disproof of this possibility, but it is unlikely that the intrathyroidal deiodination cycle is sufficiently active to support such a process.

The ratio of DIT/MIT was lower in mitochondrial than in the supernatant fraction, as reported by Tong (19), but the significance of this observation is not clear.

Inability to demonstrate iodothyronines in these studies could be due to lack of incubation for a sufficient period of time, or damage to the necessary enzymic factors of the coupling process.

It was of considerable interest that thyroglobulin was the only soluble iodoprotein detected even as early as one minute after the start of incubation. No data suggested the formation of a soluble polypeptide precursor of thyroglobulin.

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THE EARLY STAGE OF THYROID HORMONE FORMATION: STUDIES ON RAT THYROIDS *IN VIVO*[1]

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ABSTRACT

Labeled components of thyroids from normal and iodine-deficient rats have been studied 2 minutes to 24 hours after subcutaneous injection of I^{131}.

Normal glands collected 1% of dose at 120 minutes, whereas the iodine-deficient group collected 12%. Both groups accumulated I^{131} in excess of binding during the early minutes of observation. Transfer of PBI^{131} from particulate to supernatant fraction was not proved. However, in studies on the distribution of total gland homogenate PBI^{131} between supernatant fraction (soluble cell proteins and colloid) and particulate fractions (nuclear, mitochondrial, and microsomal), it was observed in some groups of animals that the proportion of PBI^{131} present in the supernatant fraction increased rapidly during the first 20 minutes, and then plateaued.

MIT and DIT were present within 2 minutes of labeling, and in hyperplastic glands thyroxine was present after 14 minutes. The ratio of DIT/MIT was reduced by iodine deficiency, but in all groups the ratio remained relatively constant during the experiment.

Thyroglobulin, and, in iodine-deficient glands a second distinct protein, were the only soluble iodinated proteins detected.

FEW data are available regarding thyroid hormone formation during the first 1–2 hours after the administration of tracer iodide. In a preceding study (1) using sheep thyroid slices labeled *in vitro* with I^{131}, it was observed that iodide accumulation exceeds binding during the first four hours of incubation, that monoiodotyrosine and diiodotyrosine are both present within 2 minutes of incubation, and that thyroglobulin appears to be the only soluble iodinated protein formed. An early period of accumulation of protein-bound I^{131} in the insoluble cell particles with subsequent transfer to the soluble fraction of the cell was not excluded. Related observations have been made on rat thyroids during the following *in vivo* studies.

METHODS

Seven groups of male Charles River rats were used (Table 1). Roman numerals appear in text and figures to identify the groups studied. The animals were maintained on

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TABLE 1. PREPARATION OF ANIMALS FOR I^{131} STUDIES

Group	Diet	Time on diet	Other treatment	Number of rats per observation point
I	Remington	4 weeks	None	2
II	Purina	3 weeks	None	2
III	Purina	6 weeks	None	2
IV	Remington	6 weeks	0.1% propylthiouracil in diet; drug cancelled 2 days before experiment	2
V	Purina	4 weeks	None	2
VI	Purina	12 weeks	None	3
VII	Remington	12 weeks	None	3

a Purina laboratory chow or Remington diet as indicated. In one instance 0.1% propylthiouracil was added to the latter diet until two days before the I^{131} studies were conducted. Within each group, the thyroids of two or three animals were pooled for each observation point.

The animals were injected subcutaneously with 50–100 μc carrier-free I^{131}, and killed by a blow on the head at the desired interval following injection. The thyroid glands were then rapidly removed and immediately homogenized in ice-cold 0.25 M sucrose. Subsequently a portion of the homogenate was separated into nuclear, mitochondrial, microsomal, and soluble (supernatant) fractions, as described previously (2). The nuclear fraction was washed twice by resuspension and resedimentation. The mitochrondrial and microsomal pellets were not washed. The pellets were dispersed in 0.25 M sucrose and fractions were taken for determination of I^{131}, PBI^{131} (protein-bound iodide^{131}), and protein content. I^{131} was separated from PBI^{131} by direct paper chromatography (3). Iodinated amino acid composition of protein was studied by chromatography after enzymatic digestion (4). Methods of analysis are described in the preceding report (1). Electrophoresis in starch gel was done by the method of Smithies (5).

RESULTS

Thyroidal accumulation of I^{131}

Thyroidal uptake of injected I^{131} in rats on a regular diet was approximately 1% of dose at 120 minutes and reached 2–4% at 24 hours. In rats on a Remington diet, uptake reached 12% at 60 minutes, and as high as 44% at 24 hours.

Organic binding of iodide

In rats on a regular diet, less than one-half of the collected iodide was protein-bound within two minutes after injection (Fig. 1). The proportion of trapped to total iodine rapidly decreased, and by 20 minutes in three of four experiments, 84–95% of collected iodide was bound. Free iodide constituted 1–2% of total thyroidal iodide at 24 hours. In one group of the normal animals, binding was considerably slower than in the other three. The only recognized difference among these animals was that the slowly binding group was eight weeks older when studied than the others.

In two groups of rats consuming an iodine-deficient diet prior to the

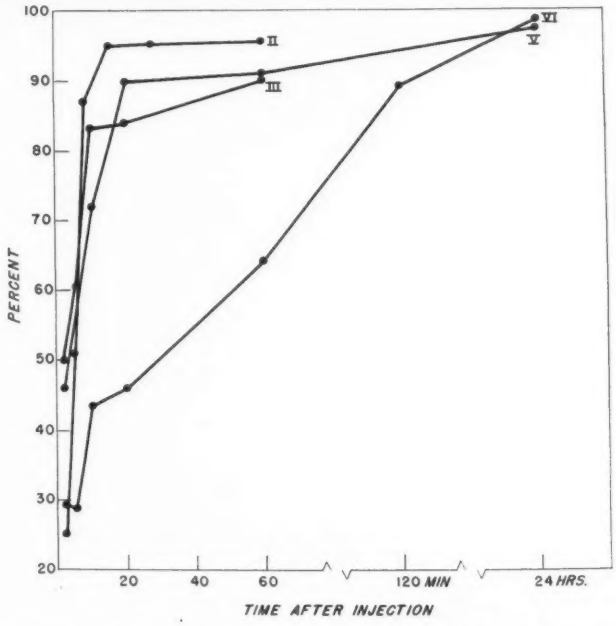

FIG. 1. Percent of accumulated thyroidal I^{131} present in bound form (ordinate) is plotted for various intervals after injection. The animals were on an iodine-sufficient diet. During the first 20 minutes a large proportion of collected I^{131} was not bound as PBI^{131}.

experiments, a higher proportion, 71–90% of trapped iodide was bound within two minutes after injection of iodide. Within five minutes bound iodine was 97–98% of total thyroidal iodine (Fig. 2). Binding was slower in one group maintained on an iodide-deficient diet and propylthiouracil (Fig. 2).

Distribution of PBI^{131} in sub-fractions of homogenized thyroids

Distribution of PBI^{131} between cell particulate elements (nuclear, mitochondrial, and microsomal fractions) and supernatant (soluble cell proteins and colloid) fraction was recorded in four groups of animals on a normal diet and in three groups of iodide-deficient animals. These studies are subject to errors derived from (1) the low counting rates in the early time intervals, and (2) differences in extent of homogenization. Variations in the extent of homogenization would alter the proportion of PBI^{131} and protein appearing in the particulate fractions and would especially affect the nuclear fraction, which contains unbroken or poorly homogenized cells.

In two groups of iodide-sufficient animals, Groups III and VI, 60.5 and 82.0% of total slice PBI^{131} was present in the soluble fraction 2 minutes

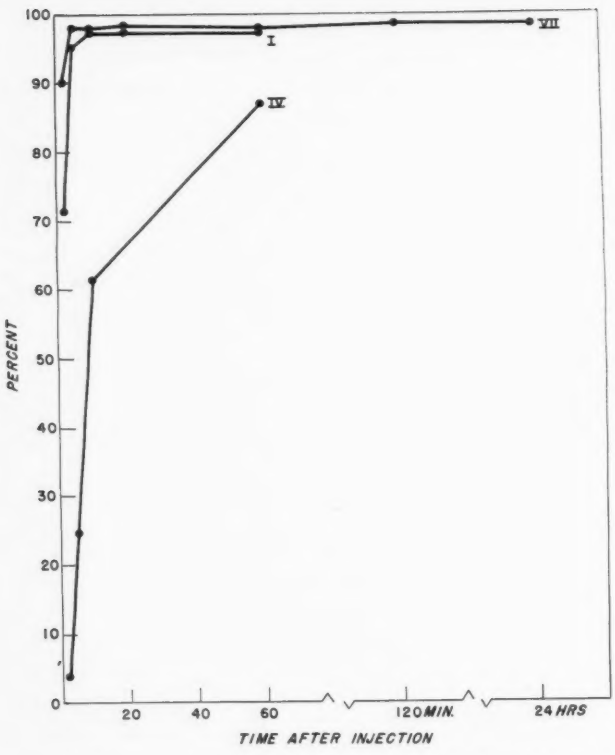

FIG. 2. Percent of accumulated thyroidal I^{131} present in bound form (ordinate) is plotted for various intervals after injection. The animals were on a Remington diet. A larger proportion of total I^{131} is present as PBI^{131} at all intervals in Groups I and VII, than in animals on a normal diet. Group IV had received propylthiouracil until 48 hours prior to the injection of I^{131}.

after injection of I^{131}. This proportion increased to 93% at 5 minutes (Fig. 3). In two other groups, II and V, there was little difference in the proportion of PBI^{131} in the supernatant fraction at early time intervals as compared to later time intervals.

In two of three groups of animals on a Remington diet, there was an increase in the proportion of total homogenate PBI^{131} in the supernatant fraction during the time interval from 2 minutes to 24 hours (Fig. 4). In Groups IV and VII the major increase occurred within the first 20 minutes.

Distribution of PBI^{131} in cell particulate fractions and supernatant fraction over the entire observation period is detailed in the accompanying chart for Group III (Fig. 5). Proportional distribution of total PBI^{131} in each fraction is diagrammed. Although total PBI^{131} content in each fraction

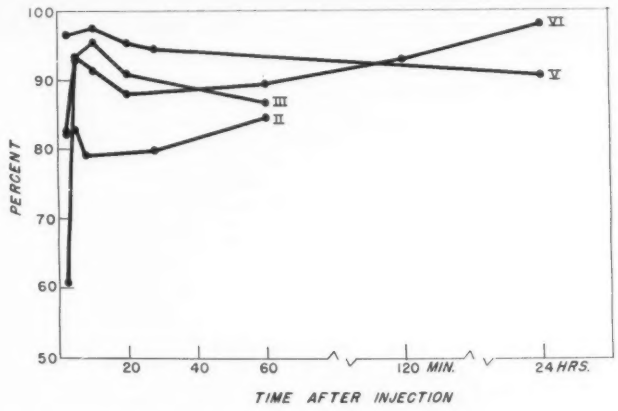

FIG. 3. In Figures 3, 4, and 5, percent of total gland homogenate PBI^{131} present in nuclear, mitochondrial, microsomal, or supernatant fractions is plotted on the ordinate against time after injection of I^{131} on abscissa. In this figure percent of total slice homogenate PBI^{131} appearing in supernatant fractions derived from the homogenates is shown for 4 groups of animals on an iodine-sufficient diet. In Groups III and VI a significant increase in this fraction occurred during the first 20 minutes after injection of I^{131}.

continually increased during each study, there was no evidence of a progressive, relative accumulation of PBI^{131} in any particulate fraction, but as described above, in several studies there was relative accumulation in the supernatant fraction.

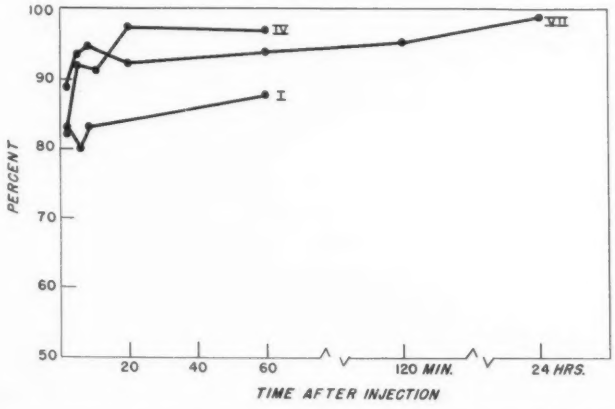

FIG. 4. Construction of chart as in Fig. 3. In this figure percent of total gland homogenate PBI^{131} appearing in supernatant fractions derived from the homogenates is shown for 3 groups of animals on an iodine-deficient diet. In Groups IV and VII there was an increase in this fraction during the first 20 minutes.

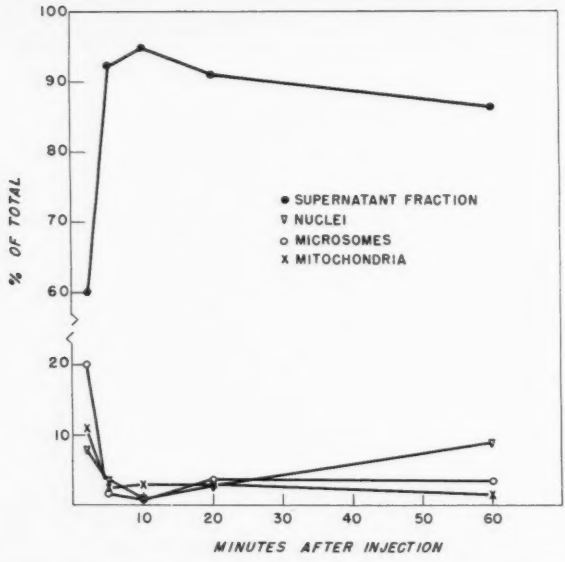

FIG. 5. Percent of total gland homogenate PBI^{131} present in nuclear, mitochondrial, microsomal, and supernatant fractions at various time intervals after injection of I^{131} is shown for Group III, rats on Purina diet. There is marked increase in the proportion of total PBI^{131} present in the supernatant fraction during the first 5 minutes.

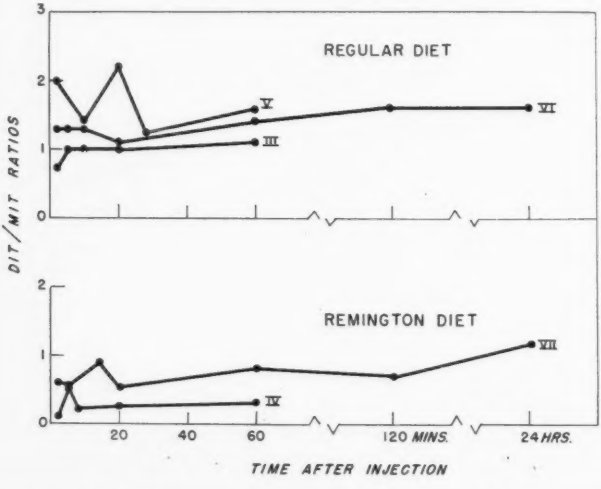

FIG. 6. The labeled DIT/MIT ratios derived from ascending chromatography of pancreatin digests of homogenate supernatant fractions is shown for three groups of animals on an iodine-sufficient diet, and two groups of iodine-deficient animals. In all groups except VII, the ratio remained essentially constant over the period observed.

TABLE 2. ALIQUOTS OF WHOLE HOMOGENATE AND EACH HOMOGENATE FRACTION FROM ANIMALS OF GROUP VII (REMINGTON DIET) WERE CHROMATOGRAPHED AFTER PANCREATIN DIGESTION

	Time	Origin	DIT	MIT	I	T_4	T_3
Whole homogenate	2 min.	8.8	28.5	47.0	9.3	1.2	1.0
	5 min.	13.4	38.9	37.6	7.3	1.0	0.8
	10 min.	8.1	32.2	48.2	5.1	0.8	0.5
	20 min.	7.2	29.2	53.6	4.8	1.4	0.4
	60 min.	12.3	34.7	40.0	4.3	1.8	0.7
	120 min.	9.1	39.0	37.3	6.1	2.5	1.5
	24 hr.	11.2	23.9	27.8	10.9	11.5	6.3
Nuclei	2 min.	3.7	17.0	22.9	41.5	9.1	1.7
	5 min.	12.5	23.7	49.3	8.8	1.0	0.4
	10 min.	15.0	29.1	41.0	6.2	1.3	0.2
	20 min.	16.4	9.8	57.8	7.3	1.5	0.8
	60 min.	16.5	15.9	42.4	7.1	2.5	3.1
	120 min.	16.7	31.9	33.2	7.3	2.0	0.4
	24 hr.	17.3	29.1	25.0	14.5	3.5	3.6
Mitochondria	2 min.	16.5	15.9	42.4	7.1	2.6	3.1
	5 min.	12.0	26.0	48.9	6.9	0.8	0.6
	10 min.	11.7	25.4	49.6	7.0	0.5	0.5
	20 min.	12.8	21.4	52.6	6.6	1.1	0.8
	60 min.	13.0	31.6	35.1	9.3	1.5	1.5
	120 min.	13.2	25.7	40.8	7.2	3.3	1.6
	24 hr.	16.3	17.3	25.7	12.1	9.3	4.4
Microsomes	2 min.	14.7	13.8	44.3	5.1	2.2	6.5
	5 min.	14.4	20.1	52.9	6.7	1.3	1.6
	10 min.	10.6	28.0	42.9	5.7	2.0	1.7
	20 min.	12.7	19.7	52.4	5.7	1.7	1.4
	60 min.	13.6	38.0	33.3	7.6	1.0	1.6
	120 min.	17.0	26.2	33.5	7.8	2.6	3.1
	24 hr.	14.4	25.6	22.5	13.7	5.5	4.0
Supernatant fraction	2 min.	12.2	25.2	39.6	11.5	2.8	2.1
	5 min.	6.5	29.9	54.9	4.7	0.8	0.4
	10 min.	7.4	40.5	43.0	3.8	1.5	0.7
	20 min.	7.3	23.3	49.0	3.5	1.7	0.5
	60 min.	8.8	33.7	42.6	4.6	4.6	1.6
	120 min.	8.1	32.6	44.2	4.1	4.9	1.4
	24 hr.	12.0	26.2	22.0	5.5	17.6	6.9

The proportion of total homogenate fraction I^{131} present in the zone of added stable markers is given for each observation period following injection.

The ratio of DIT to MIT in digests of supernatant fractions of thyroids

The ratios of DIT to MIT in digests of the supernatant fraction from five groups of rats appear in the accompanying chart (Fig. 6). In three groups of animals on a regular diet the ratio was 1 or more. Although the points are somewhat erratic, there was no indication of significant change in DIT/MIT ratio during the period of observation. DIT was present in the digests as early as MIT was observed.

In one group of animals on a low iodine diet and propylthiouracil, the ratio of DIT/MIT was 0.1–0.3 during a period of 0–60 minutes. In one group on an iodine-deficient diet alone, the ratio was approximately 0.5 at the earliest time period and gradually rose to 1.2 at 24 hours.

The ratio of DIT/MIT in each sub-fraction of the homogenate resem-

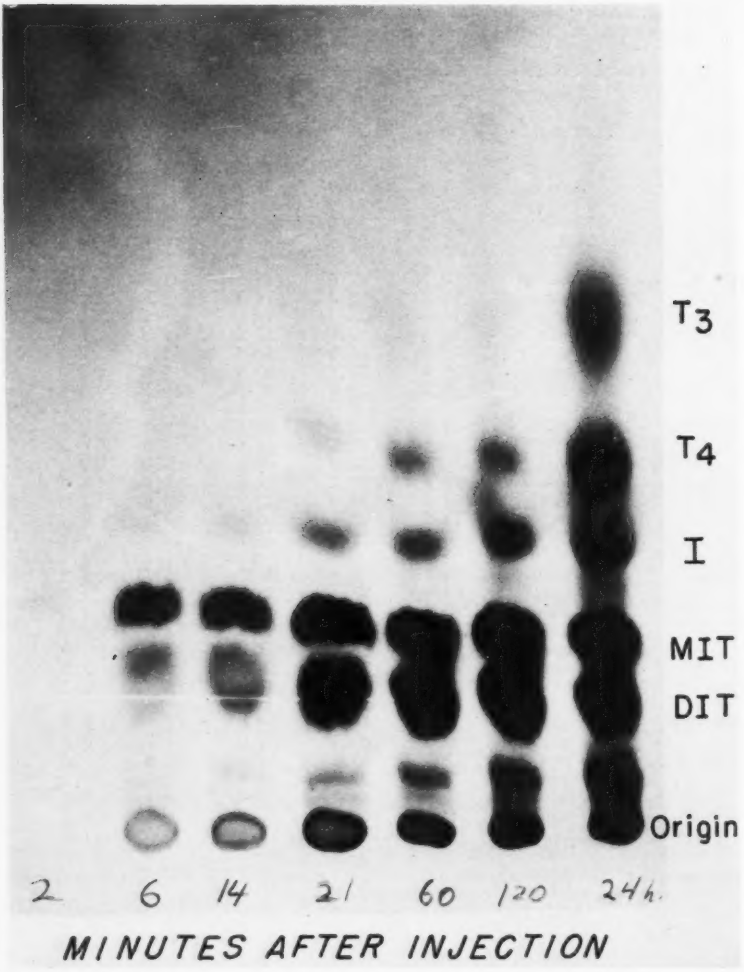

FIG. 7. Radioautograph of an ascending chromatogram prepared from pancreatin digested homogenates. The animals, from Group VII, were killed at the stated intervals after injection of I^{131}. Although not well portrayed in this print, MIT and DIT were clearly visible at 2 minutes, T4 (thyroxine) and T3 (triiodothyronine) at 14 minutes.

bled that in the whole homogenate (Table 2). However, the DIT/MIT ratio in the mitochondrial fraction was slightly lower than in the supernatant fraction in rats on the iodine-sufficient diet.

The chromatograms clearly contained mono- and diiodotyrosine, as shown in the accompanying radioautograph prepared from direct chromatograms of digests (Fig. 7). Although some radioactivity was always recorded in the thyroxine and triiodothyronine zones, radioautographs did

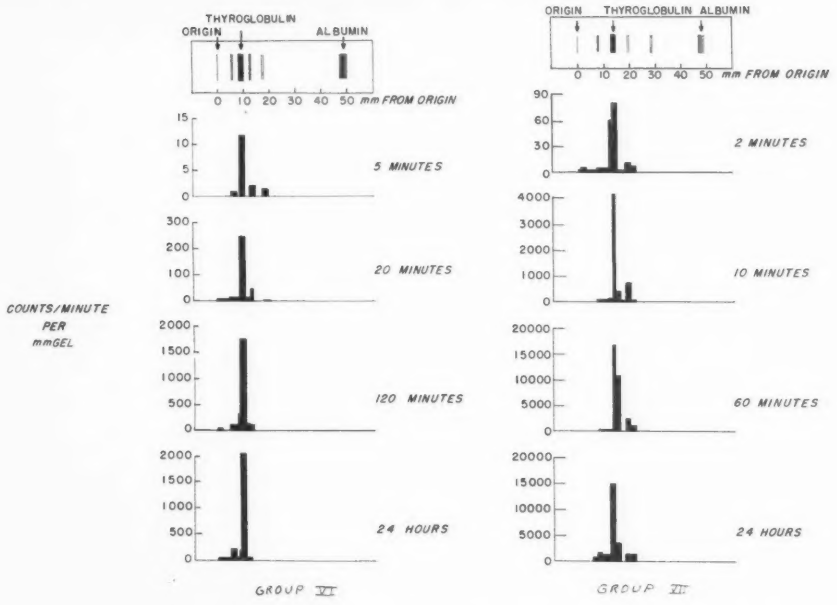

FIG. 8. Aliquots of homogenate supernatant fractions from Group VI (Purina diet) and Group VII (Remington diet) were subjected to electrophoresis in starch gel. Diagrams of the gels appear above the graphs, with protein bands darkened to depict their relative intensity of staining. The anodal migration of the protein components is depicted on the abscissa, and PBI^{131} content of the proteins on the ordinate. In glands from iodine-sufficient animals PBI^{131} was found consistently only in the thyroglobulin band. Smearing of some I^{131} around thyroglobulin, not corresponding to distinct protein bands, was sometimes observed. In iodine-deficient animals a second protein moving ahead of thyroglobulin was also iodinated.

not clearly demonstrate the presence of these amino acids until 60 minutes had elapsed in animals on the regular diet, or 14 minutes in the iodine-deficient animals. In one group of iodine-deficient animals the thyroxine /triiodothyronine ratio remained approximately 3:1 from 14 minutes to 24 hours, in contrast to a ratio of 4–5:1 in one group of animals on the normal diet.

Soluble proteins iodinated

Protein components of the supernatant fractions were examined by starch gel electrophoresis. In thyroids from rats on a normal diet, PBI^{131} was consistently found only in the thyroglobulin band (Fig. 8). In starch gel electrophoresis of supernatant fraction proteins from animals that had consumed iodine-deficient diets (with or without propylthiouracil), thyroglobulin was iodinated, as well as a protein which moved anodally ahead of thyroglobulin (Fig. 8). In each instance thyroglobulin was iodinated

within two minutes after injection of I^{131}. In the animals on the iodine-deficient diet, there was no alteration in the proportion of PBI^{131} in the thyroglobulin to that in the pre-thyroglobulin component with elapse of time after injection of $iodide^{131}$.

DISCUSSION

Thyroidal uptake of I^{131} in animals on a regular diet was exceedingly low. With a diet deficient in iodide, it was possible to elevate the uptake to approximately 40% of injected dose at 24 hours. In animals on a regular or a Remington diet, the thyroid gland accumulated iodide considerably in excess of binding during the first 10 to 20 minutes following injection of I^{131}.

It is probable that active trapping continually provides a pool of free iodide within the thyroid. However, the proportion of free iodide gradually diminishes as the I^{131}-label accumulates in the thyroglobulin, so the phenomenon is not obvious after 20 minutes. Trapping of iodide in excess of binding has been observed by other investigators (6). In the animals on an iodide-deficient diet, although uptake of I^{131} was greatly augmented, the trapping process did not outstrip the binding process, since a greater proportion of iodide was bound in their thyroids at each interval of observation. The reduced binding observed in Group VI may have resulted from residual propylthiouracil, although this drug had been withdrawn from their diet 48 hours prior to the experiment (7).

Studies on distribution of PBI^{131} in homogenate sub-fractions did not clearly establish a period of accumulation of PBI^{131} in the particulate fraction with subsequent transfer to the soluble fraction. This trend was suggested in two of the groups on a regular diet and two groups on an iodide-deficient diet, but the evidence is not conclusive. The observed alterations in proportional distribution of PBI^{131} were largely completed in the first 20 minutes of the experiments. After this time 85–97% of total PBI^{131} was in the supernatant fraction.

It is apparent that iodination of particulate fraction protein goes on at the same time as soluble fraction protein. It is by no means clear that the particulate fraction PBI^{131} represents a precursor of soluble fraction PBI^{131}, although this is a logical hypothesis. On the contrary, it is equally possible that PBI^{131} in the particulate fraction is an end product, not a precursor. In this case an alteration in the ratio of PBI^{131} in the particulate to supernatant fractions might represent simply a saturation of binding sites in the particulate fraction, rather than a transfer of particulate to soluble fraction protein. Further study is needed on this point.

Studies on DIT/MIT ratio in digests of fractions of the homogenates demonstrated the presence of DIT as early as MIT was formed. Furthermore, there was little alteration in the ratio of DIT/MIT during the observation periods. Similar results have been reported by Bois and Larsson

(8) and by Kobayashi and Gorbman (9). The ratio of DIT/MIT was above 1 in animals on a regular diet and reduced (.3 to 1.2) in animals on an iodine-deficient diet with or without propylthiouracil. A decrease in this ratio in response to iodide deficiency had been previously demonstrated by Leloup and Lachiver (10) and by Querido *et al.* (11). These observations suggest that MIT is not simply a precursor of DIT and that there is probably considerable specificity in the extent of iodination of tyrosyl groups in thyroglobulin.

In studies on soluble iodinated proteins formed in animals on a normal diet, only thyroglobulin was apparent. There was no evidence for a soluble precursor polypeptide or protein. In animals on an iodide-deficient diet, a second iodinated protein was detected. However, the proportion of total PBI^{131} in this as compared with thyroglobulin did not change during the 24-hour observation period, and thus it cannot be considered a precursor of thyroglobulin. It is apparently formed in parallel with thyroglobulin and represents a second soluble iodinated protein, as has been described by other workers in human and animal glands (12, 13). It is entirely possible that this protein was present in the normal glands, but in such small amount as to escape detection by our methods, which would probably not detect an iodinated protein if it contained less than 2% of total soluble PBI^{131}. It is probable that the second iodinated protein appears as a response to thyrotropin stimulation, but its significance is unknown.

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THE EFFECT OF ELECTRICAL ABLATION OF THE NUCLEI HABENULAE, PINEAL BODY AND SUBCOMMISSURAL ORGAN ON ENDOCRINE FUNCTION, WITH SPECIAL REFERENCE TO THYROID FUNCTION[1]

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ABSTRACT

Although it has been proposed that the habenular nuclei may be a primary center in the control of thyrotropin secretion by the pituitary, in the present study it was found that animals with extensive damage including the habenula, pineal body and subcommissural organ developed thyroid enlargement approximately equal to that of untreated controls when fed propylthiouracil. Thyroidal radioiodine accumulation and testis and adrenal weights were not consistently affected by any of the lesions. There was no persistent change in either serum sodium and potassium concentration or water intake postoperatively.

IT HAS become increasingly evident that the hypothalamus plays an exceedingly important role in the control of pituitary secretion (1–4). The evidence suggests that hypothalamic control is mediated by way of some unknown neurohumoral material(s) which passes through the hypophyseal portal vessels from the median eminence of the hypothalamus to the anterior pituitary (5). However, it is possible that the hypothalamus is not a primary center, but merely transmits impulses from higher centers, relaying them to the pituitary by neurohumoral or nervous transmission. Several reports have indicated participation by higher centers in the control of corticotropin and gonadotropin secretion (6, 7).

The epithalamic area has recently been implicated in the control of certain endocrine functions. It has been proposed that the pineal body secretes a humor which regulates aldosterone secretion (8, 9) and that the subcommissural organ somehow controls water metabolism (10, 11). Of special interest are the reports of Mess, who states that lesions in the habenular nuclei inhibit goiter development caused either by low iodine diet or by antithyroid drugs (12) and that atrophy of the thyroid gland following exogenously administered thyroid hormone is prevented by such lesions

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(13). He concludes that the habenular nuclei are the primary center for the regulation of thyrotropin secretion, the well-known effect of hypothalamic lesions on TSH secretion being due merely to the concomitant operative damage of the hypophyseal portal vessels.

Because of the potential importance of such reports, we decided to conduct a series of experiments on the effect of epithalamic lesions in the rat.

MATERIALS AND METHODS

Sixty adult male Holtzman rats, weighing 220 to 309 gm., were employed in these experiments. Four consecutive experiments were designed, involving the following lesions: (1) Total destruction of the habenular nuclei and partial destruction of the pineal body, (2) Partial destruction of the habenula and total destruction of the pineal body, and (3) Total destruction of the habenula and subcommissural organ and partial destruction of the pineal body. The fourth experiment was to observe the effect of the same lesions as (3) after a longer period of time.

Electrolytic lesions were made bilaterally with a stereotaxic instrument. From 16 to 20 separate electrode insertions were made in each animal, and a direct current of 6 mA for 15 seconds was utilized with each insertion. Beginning 8 days postoperatively, the animals were fed a low iodine diet with or without 0.15% propylthiouracil (PTU). Four or nine hours before the autopsy, 0.1 μc of radioiodine was injected intraperitoneally. On the 10th or 20th day of the low iodine diet, the animals were autopsied. The thyroids, adrenals and testes were dissected and weighed on a torsion balance. The brains were fixed in 10% formalin, serial sections were made at 10μ and every 10th section was mounted and stained with hematoxylin-eosin.

Organ weight/body weight was calculated in reference to the weight of the animals at the start of the low iodine diet, because PTU-fed animals, particularly those with lesions, lost weight during the experiment.

Serum sodium and potassium concentrations were determined by flame photometry.

RESULTS

The effect of ablations of the nuclei habenulae, pineal body and subcommissural organ on endocrine function

The effect of total destruction of the nuclei habenulae and partial destruction of the pineal body on the weight of thyroids, adrenals and testes is shown in Experiment I of Table 1. It will be seen that the size of the goiter in the group with lesions was somewhat smaller than that in the control group, but not significantly so. Testes and adrenal weights also showed no significant differences. The results were not sufficiently clear cut to allow an interpretation.

The data from the second experiment, using animals with partial destruction of the nuclei habenulae and total destruction of the pineal body are shown in Experiment II of Table 1. Goiter did develop in the lesion bearing animals fed PTU but not in those fed a low iodine diet. The lesion alone did cause a significant increse in adrenal weight which may have been accentuated by PTU, and in addition it appears that there may be an effect by the lesion alone on testis weight.

The next step was to see whether total destruction of both the nuclei

TABLE 1. THE EFFECT OF EPITHALAMIC LESIONS ON CERTAIN ENDOCRINE GLANDS

Experiment and Group		Number of animals	Days of exp.	Initial Body wt. (g.)	Thyroid wt. mg./100 g. B.W.	Adrenal wt. mg./100 g. B.W.	Testis wt. g./100 g. B.W.	Thyroidal I^{131}% uptake
I. Total destruction of habenula; partial destruction of pineal	L+PTU	6	9	245 ± 9	13.00 ±0.84	18.31 ±1.05	1.30 ±0.03	
	PTU cont.	4	9	251 ± 8	15.41 ±1.56	16.96 ±0.77	1.31 ±0.03	
II. Partial destruction of habenula; total destruction of pineal	L only	4	9	291 ± 3	8.92 ±0.23	17.20 ±1.10	1.27 ±0.04	6.7 ±0.7
	L+PTU	4	9	258 ±10	16.64 ±0.75	19.06 ±1.07	1.28 ±0.07	
	Untreated	4	9	302 ± 8	8.26 ±0.26	14.09 ±0.48	1.08 ±0.08	8.6 ±0.5
	L—(L+PTU)				−7.72 <0.1%	—	—	
	L—Untreated				—	+3.11 <5%	+0.19 <10%	
	(L+PTU)—Untreated				+8.38 <0.1%	+4.97 <1%	—	
III. Total destruction of habenula and subcommissural organ; partial destruction of pineal	L only	6	9	241 ±10	9.98 ±0.85	18.34 ±0.79	1.21 ±0.06	28.0 ±1.5
	L+PTU	6	9	232 ± 5	13.01 ±0.93	16.34 ±0.71	1.30 ±0.08	
	PTU cont.	5	9	280 ± 5	16.64 ±0.91	14.04 ±0.96	1.10 ±0.04	
	Untreated	5	9	272 ± 6	9.24 ±0.68	17.08 ±1.02	1.17 ±0.03	27.7 ±1.3
	L—(L+PTU)				−3.03 <5.0%	+2.00 <10%	—	
	L—PTU cont.				−6.66 <0.1%	+4.30 < 1%	—	
	L—Untreated				—	—	—	
	(L+PTU)—PTU cont.				−3.63 <5.0%	+2.30 <10%	+0.20 <10%	
	(L+PTU)—Untreated				+3.77 <1.0%	—	—	
	PTU cont.—Untreated				+7.40 <0.1%	−3.04 <10%	—	
IV. Same as III	L+PTU	7	19	208 ± 5	21.30 ±0.74	16.04 ±1.02	1.25 ±0.10	
	PTU cont.	5	19	270 ±15	19.24 ±1.69	13.42 ±0.74	1.16 ±0.07	
	Untreated	4	19	278 ± 9	10.70 ±0.72	14.26 ±2.53	1.04 ±0.11	
	(L+PTU)—PTU cont.				—	+2.62 <10%	—	
	(L+PTU)—Untreated				+10.60 <0.1%	—	—	
	PTU cont.—Untreated				+8.54 <1.0%	—	—	

Results expressed as mean ± standard error of mean. L = lesion. Observed significant differences are shown by their P values. Calculation for

$$\text{'t'} = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{SE_1^2 - SE_2^2}}.$$

I^{131} was injected 4 hours before autopsy in Exp. II, and 9 hours before autopsy in Exp. III. Organ weights are expressed in relation to body weight at the initiation of the low iodine diet.

habenulae and the subcommissural organ, with partial destruction of the pineal body, would produce a more clear-cut suppression of thyrotropin secretion. As shown in Experiment III of Table 1, goiter again developed in PTU-fed animals with lesions, and its size was very significantly smaller than that of the PTU-fed animals without lesions. Thyroid weight and radioiodine uptake in the animals with lesions did not vary significantly from the untreated group. In contrast to Experiment II, this lesion by itself does not seem to have had any significant effect on adrenal weight, but it does appear to counteract to a significant extent a marked decrease

TABLE 2. SERUM ELECTROLYTE CONCENTRATION AFTER EPITHALAMIC LESIONS

Experiment and group		Number of animals	Duration of experiment (days)	Serum Na M.eq./L	Serum K M.eq./L
II	L	4	9	144.7 (±1.1)	5.0 (±0.2)
	Control	4	9	148.3 (±1.0)	5.1 (±0.1)
III	L	6	9	146.5 (±0.9)	6.6 (±0.1)
	Control	5	9	150.1 (±2.2)	6.7 (±0.1)
IV	L	7	19	139.0 (±0.3)	6.6 (±0.2)
	Control	5	19	139.9 (±0.6)	6.0 (±0.1)

Results expressed as mean ± standard error of mean. L = animals with brain lesions fed PTU. Control = non-lesioned animals fed PTU. Experimental groups same as Table 1.

in adrenal weight caused by PTU. There is again a rather weak indication of some effect on the testes.

These results seemed to bear out Mess' work to some extent, but we felt it would be worthwhile to go even further by repeating the last experiment, allowing 19 days, rather than nine, before autopsy. As indicated in Experiment IV of Table 1, at the end of 19 days, the significant effect of the lesion on PTU-induced goiter was not present and, if anything, the effect seemed to be heading in the other direction. In addition the significant differences in adrenal weights seem to have disappeared almost entirely, along with any effect on testis weights. Histologic observation indicated that lesions were present in all the desired areas and in some adjacent areas as well.

The effect of ablation of the habenular nuclei, pineal body and subcommissural organ on electrolyte concentration and water intake

The final concentration of serum sodium and potassium was the same in the intact animals and those with lesions in each of the experiments, although there was some variation in the values from group to group (Table 2). Water intake was markedly suppressed for the first two days after the operation but rapidly returned to control levels; no difference was evident one week after the operation.

DISCUSSION

It has been suggested that some part of the central nervous system other than the hypothalamus may influence the secretion of certain pituitary hormones, among them thyrotropin. However, extensive studies by Greer and co-workers have failed to detect important participation of centers other than the hypothalamus in the regulation of thyrotropin secretion (14–16). Further studies in this laboratory have indicated that the anterior hypothalamic "thyrotropin" area is sensitive to local changes of thyroid hormone concentration and may thereby act as a "thermostat" in the servo mechanism which maintains a stable level of circulating thryoid hormone (17–19).

Mess found that lesions made in the nuclei habenulae inhibited goiter development caused either by low iodine diet or by antithyroid drugs (2). We found this to be true over a short period of time, although in our rats the effect of the lesion was not as marked as that found by Mess. However, after an extended period of time, we found no significant difference in the goiter size of PTU-fed animals whether lesions were present or not. This, in addition to the fact that animals with the habenula completely destroyed showed no deviation from normal radioiodine uptake, raises a serious question about the possible role of the habenula in regulating the secretion of thyrotropin by the pituitary. It should be mentioned here that animals with lesions showed a marked loss of appetite, resulting in a pronounced decrease in body weight, during the first few days of the PTU diet. It is possible that this loss of appetite influenced the goiter development, either by causing malnutrition or by reducing the intake of PTU.

Farrell *et al.* (8, 9) recently proposed the hypothesis that the pineal body secretes glomerulotropin which stimulates the secretion of aldosterone by the adrenal cortex. Although this suggests that the pineal body may control electrolyte metabolism, in our experiments the final concentrations of serum sodium and potassium were not significantly different as a result of the presence of lesions.

Gilbert (10, 11) has suggested that the subcommissural organ, which has long been suspected from histological studies of being an endocrine organ, may control water metabolism in the rat by controlling water intake. However, he states that rats die from dehydration after electrical ablation of the subcommissural area. This was not the case in our experiments.

Testicular changes are really too doubtful to be commented on. Although alterations of the adrenal weight might bear further investigation, the results are so inconstant as to make it unlikely there is any real effect of the lesions on ACTH release.

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CHANGES IN PLASMA PROTEIN-BOUND IODINE AND THYROXINE-LIKE ACTIVITY OF FECES OF DAIRY COWS BEING FED L-THYROXINE

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ABSTRACT

The effect of L-thyroxine, in relation to dose-level and length of time fed, upon plasma PBI and fecal thyroxine was determined in lactating cows. Two cows were fed varying daily dosages of L-thyroxine, with the level changed weekly. They were started at 60 mg. per day for the first week, with 80, 100, 120, 140, 160, 100, 80, and 80 mg. following in consecutive weeks. PBI concentration in both cows reached a maximum above initial levels the second week when 80 mg. were fed. The actual excretion of equivalent L-thyroxine in the feces generally increased as dose-level increased. However, the percentage of dose excreted decreased until 120 mg. of thyroxine were fed daily and then tended to remain constant through the 160 mg. daily dose.

The changes of PBI concentration in two cows fed 110 mg. of L-thyroxine daily for 15 weeks followed parallel patterns, with a declining trend as treatment continued. In one cow the actual excretion of thyroxine and the percentage of dose excreted decreased as treatment progressed. The other cow showed approximately the same degree of thyroxine excretion throughout the treatment period.

The results indicate that the cow's body trys to rid itself of excess thyroxine. It appears that a metabolic or gastrointestinal destruction and/or degradation mechanism is stimulated to be more effective by increasing doses of thyroxine and extended periods of thyroxine feeding. Because of this the plasma PBI level resulting from feeding thyroxine will not be constantly proportional to the dose.

THE galactopoietic response of dairy cows to the continued feeding of thyroprotein or L-thyroxine is greatest at the beginning of the treatment period, then it gradually diminishes until nearly normal lactation levels are resumed after about ten weeks of treatment (1, 2). At the time the lactation stimulus is waning, other signs of hyperthyroidism such as increased heart rates and decreased body weights also begin to turn toward the normal condition in spite of continued thyroxine feeding (3, 4). The nature of this homeostatic response has not been determined. Studies of the metabolic destruction of injected thyroxine in dogs by O'Neal (5) and of the excretion of injected thyroxine in rats by Taurog *et al.* (6) have indicated that excessive thyroxine in the system stimulates the degrada-

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tion and excretion of thyroxine. The purpose of this investigation was to determine if there was an increased rate of peripheral metabolism and/or an increased rate of gastrointestinal destruction of thyroxine in cows fed large doses of thyroxine for extended periods.

MATERIALS AND METHODS

The investigation consisted of feeding daily controlled doses of L-thyroxine to dairy cows for different periods. At intervals before, during and after the treatment periods blood samples and 24-hour feces collections were obtained. Plasma protein-bound iodine (PBI) determinations were made in duplicate weekly to detect probable changes in circulating thyroxine. The thyroxine equivalent activity excreted in the feces was determined and compared with PBI levels. The steady heart rate of each cow was determined each week when the cows were at rest before milking.

The thyroxine used for feeding was crystalline sodium L-thyroxine pentahydrate (Baxter Laboratories). This was powdered in corn starch and thoroughly dispersed in a mixture of corn starch and soybean oil meal to give a final concentration of one percent sodium L-thyroxine (corrected for moisture). Doses to be fed were weighed accurately into envelopes so that half of the daily allowance for each cow was fed mixed with the grain feed at each twice-daily feeding of grain. This same stock source of sodium L-thyroxine was also used for preparing thyroxine bearing rations used in the chick assays.

Four lactating grade dairy cows were used in the excretion studies. Two 6-year old Guernsey twins (Nos. 9 and 10) were used to study the effects of continuous feeding of the same dose. One 7-year old Jersey (No. 23) and one 2-year old Holstein (No. 51) were used in a study of the effects of different dose levels. In addition, PBI studies were made with 4 pairs of identical twin cows in a spring to summer period and with 4 groups of 6 cows each which were fed different daily doses of thyroxine in a fall to winter period.

The cows were kept in a conventional stanchion type barn. Their roughage feed consisted mostly of alfalfa and grass mixed hay, but it was not uniform in quality throughout the study. Cows 9 and 10 were also allowed some grass pasture. The concentrate fed was a home-mixed ration containing about 15% protein. It was fortified only with salt (NaCl) and steamed bone meal as minerals.

Thyroxine feeding of cows 9 and 10 started at 140 and 175 days of lactation, respectively. They were each fed 110 mg. sodium L-thyroxine daily for 13 consecutive weeks, and then the thyroxine was gradually withdrawn from the ration over a 2-week period. Feces collections were made one week before and two weeks after the thyroxine feeding period, and at 3, 8, and 12 weeks after thyroxine feeding started. These collections roughly coincided with the period of peak lactation stimulus (3 weeks), during the period of moderately reduced stimulus (8 weeks), and the period of slight or no stimulus (12 weeks) as had been observed in previous trials.

Cows 23 and 51 were started at 132 days of lactation on 60 mg. sodium L-thyroxine daily. This level was fed for one week, and then it was increased for successive weekly feedings to 80, 100, 120, 140, and 160 mg. This was followed by one week at 100 mg. and two final weeks at 80 mg. sodium L-thyroxine daily. Blood and feces collections were made after each dose had been fed for 6 days, as well as one week before and three weeks after thyroxine feeding.

The total 24-hour feces collection was weighed. Approximately one-third was then weighed into shallow enameled pans and dried at 45° C in a forced-air drying oven. The air-dry weight was determined after equilibration in a store room, and the feces were stored dry until assayed.

The feces were assayed for thyroxine equivalent activity by the goiter-prevention method described by Dempsey and Astwood (7) and Monroe and Turner (8). Each dried

feces sample was mixed into a commercial turkey poult feed at three different concentrations. Each mixture bearing a different concentration of feces and 0.15% thiouracil was fed to a group of 20 cockerel chicks. The test mixtures were fed for 21 days in rooms controlled at 70 to 75° F temperature. No hover heat was used the last 7 days of the test. The thyroid glands of each chick were weighed and the group average weights of the thyroids per 100 gm. chick body weight were determined. The concentration of feces necessary to produce normal-sized thyroids was determined by interpolation. The thyroxine equivalent of the feces was calculated from groups fed thyroxine standards along with 0.15% thiouracil. These indicated the amount of sodium L-thyroxine necessary to produce normal thyroids. Control groups (no feces fed) and thyroxine standard groups of chicks were included in each group of assays made at one time.

Plasma PBI was determined from heparinized blood according to the method of Brown *et al.* (9) as revised by Lennon and Mixner (10) and modified for use with an Evelyn photoelectric colorimeter.

RESULTS

Continuous feeding of a constant dose of thyroxine. The PBI and fecal thyroxine values observed in cows 9 and 10 are shown in Figure 1 along with a plot of their weekly body weights. These data show that PBI tended to reach maximum levels in the first 5 weeks of feeding thyroxine, and thereafter it gradually declined about 2 μg per 100 ml. during the rest of the period that 110 mg. per day of thyroxine was being fed. The one very high value for each cow about mid-way in the feeding period is assumed to be a spurious value since all of the others are fairly close to the trend line. The body weight changes indicate some tolerance of or recovery from the hyperthyroid condition after 6 to 8 weeks of thyroxine feeding.

There was not a significant amount of thyroxine-like activity in feces collected either one week before or after the thyroxine feeding period. Chick thyroids from all levels of assay from these control feces were just as large as those from chicks fed the 0.15% thiouracil ration alone. Thyroxine equivalent activity was detected in the feces during the thyroxine feeding period. This is plotted in Figure 1 as the percentage of the daily dose excreted in the feces. From cow 9 it seemed to be highest at 3 weeks and then to decline somewhat in later samples. This pattern was not observed with cow 10, however. The very low value of cow 10's feces at 8 weeks was undoubtedly influenced by mild diarrhea at that collection. The fecal thyroxine values did not increase as PBI decreased; but on the contrary, agreed reasonably closely with the trend of PBI.

Results from a comparable study of PBI changes during continuous feeding of thyroxine to lactating cows are presented in Table 1. In the trial with identical twins in which one of each pair was fed 120 mg. thyroxine daily, the trend of PBI values is roughly downward after the first sample taken at 2 weeks of thyroxine feeding. These results are similar to those with twins 9 and 10 (Fig. 1). Both twin experiments were conducted in summer. It was noted that the trend of PBI in the untreated control twins was also downward during the trial. Possibly the same factors causing a

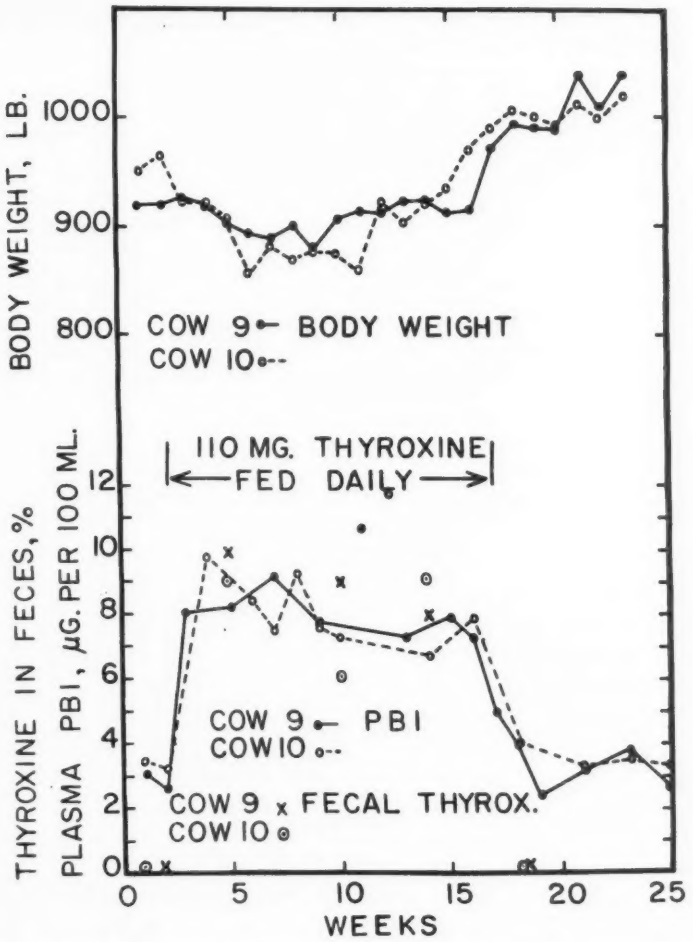

FIG. 1. The effect of daily thyroxine feeding on body weight, plasma PBI and thyroxine excretion in feces of two lactating cows.

slight decline in PBI in the control cows were affecting PBI to some extent in those which were fed thyroxine. This downward trend in PBI with continued thyroxine feeding was not observed in either the control or treated cows in Trial II (Table 1). Trial II was conducted during the late fall and winter. PBI values fluctuated in all groups during the treatment period, and they were nearly the same after 14 weeks of thyroxine feeding as after only 2 weeks. The post-treatment and pre-treatment PBI levels were also very similar in Trial II but not in Trial I. These comparisons indicate that the season of experimentation and its effect on thyroxine metabolism was affecting the results observed.

TABLE 1. CHANGES IN PLASMA PROTEIN BOUND IODINE OF LACTATING COWS FED CONSTANT DAILY DOSES OF 80, 110, 120, OR 140 MG. L-THYROXINE FOR EXTENDED PERIODS

Period and time	Trial I (summer) (Av. 4 pr. ident. twins)		Trial II (winter) (Av. 6 cows per group)			
	Control	120 mg. Thyrox.	Control	80 mg. Thyrox.	110 mg. Thyrox.	140 mg. Thyrox.
	----------		(μg PBI per 100 ml.)			
Pre-treatment						
1–2 wk.	4.9	4.7	4.1	3.8	3.3	3.9
Thyroxine fed						
2 wk.	5.2	12.9	3.4	10.0	10.9	13.1
4 wk.	4.9	10.4	3.9	9.3	9.6	13.6
6 wk.	4.7	11.1	3.6	9.5	10.3	14.4
8 wk.	4.6	11.4	3.8	9.8	11.4	13.6
10 wk.	4.2	10.0	3.9	10.3	12.7	14.7
12 wk.	3.8	11.1	3.8	10.3	10.1	16.2
14 wk.	3.9	9.6	3.6	9.5	10.8	12.8
Post-treatment						
2 wk.	3.6	3.6	4.0	3.6	3.8	4.5
4 wk.	3.3	3.8	3.6	3.3	3.4	4.1
6 wk.	3.4	3.6	3.5	3.4	3.4	3.9
8 wk.	3.3	3.5	3.3	3.1	3.3	3.6
10 wk.	3.6	3.9	3.2	3.0	3.2	3.6

Changing L-thyroxine dose weekly. The effects upon PBI and thyroxine equivalent in the feces of changing the amount of thyroxine fed to cows from week to week are shown in Table 2. Also presented in Table 2 are body weight and heart rate observations made each week as an indication of the degree of metabolic stimulation which was produced. The cows differed widely in their responses, which may have been due to age differences or to inherited metabolic differences. Cow 51 may have had higher thyroid activity than cow 23 before treatment, as evidenced by a higher

TABLE 2. THE EFFECT OF FEEDING DIFFERENT LEVELS OF L-THYROXINE UPON BODY WEIGHT, HEART RATE, PLASMA PROTEIN-BOUND IODINE AND FECAL THYROXINE IN LACTATING COWS

Week of expt.	Thyroxine fed/day	Cow 23				Cow 51			
		Body weight	Heart rate	PBI	Thyroxine in feces	Body weight	Heart rate	PBI	Thyroxine in feces
	mg.	lb.	per min.	μg %	% of dose	lb.	per min.	μg %	% of dose
1	none	1043	72	2.3	0	918	90	4.3	0
2	60	1052	70	8.1	21	911	86	14.6	15
3	80	1045	72	11.6	15	875	90	20.1	11
4	100	950	82	11.6	16	845	86	19.6	15
5	120	938	94	9.6	9	822	101	19.8	12
6	140	920	81	9.2	8	780	95	18.6	7
7	160	890	84	9.2	10	777	108	17.8	11
8	100	912	84	5.6	11	786	90	18.2	11
9	80	967	76	4.9	6	828	89	11.7	11
10	80	968	80	7.0	6	831	86	13.2	8
11	none	968	72	2.0	0	855	76	3.7	0
12	none	946	72	3.8	—	889	78	5.1	—
13	none	1008	56	3.5	0	890	80	3.8	0

heart rate and a higher PBI level than cow 23. For comparative purposes, the average heart rates of 19 similar, normal, lactating cows in gestation less than 120 days was 73.0 ± 4.1[2] from 103 different weekly observations made in summer. The average of 287 PBI values from 33 similar cows in normal condition in this herd was 3.75 ± 0.79[2] during the year previous to this investigation. Indications of possible PBI changes due to season and advancing lactation have been presented in the control groups in Table 1.

Each cow lost about 150 lbs. in body weight in the first 6 weeks of treatment, then gradually gained back about 70 lbs. by the end of treatments. This change in body weight was accompanied by increasing and then decreasing heart rate in cow 23. Heart rates also increased with the highest doses for cow 51, but generally remained high until after treatment ceased.

The PBI of both cows increased rapidly as thyroxine was fed and reached its highest values at the first week's feeding of 80 to 100 mg. daily. After this peak, PBI of cow 23 declined slightly as daily doses were increased for three more weeks, and at the second feeding of 100 mg. to 80 mg. daily it was only about one-half the former level. In cow 51 PBI values also decreased slightly after the peak values, in spite of increasing thyroxine doses, but at the second feeding of 100 and 80 mg. daily were about 90 and 65%, respectively, of the earlier levels at the same dose. Normal PBI levels were resumed in both cows within one week after stopping thyroxine feeding.

The percentage of fed thyroxine which could be detected in the feces by bioassay reached a peak in the first treatment week when only 60 mg. per day were fed. The general excretion pattern was about the same for the two cows, except that in the later stages cow 23 excreted less thyroxine activity than did cow 51. In the second period when 80 mg. daily were fed, cow 51 excreted about the same thyroxine activity as in the first 80 mg. period, but cow 23 excreted only 40% of her former level. In this respect the excretion of thyroxine activity agreed with the PBI differences between the two cows. During the earlier part of the trial, however, thyroxine activity excretion was about the same in the two cows although the PBI of cow 23 was only about one-half that of cow 51. Cow 51 had mild diarrhea during the collection when 140 mg. thyroxine per day was fed. This probably caused the low excretion value for her at that time. As noted with cows 9 and 10, there was not a detectable amount of thyroxine activity in the feces of 23 and 51 in any period when they were not being fed thyroxine.

DISCUSSION

Thyroxine may be removed from the body by metabolic destruction and excretion. Kellaway *et al.* (11) suggested that excess thyroxine stimulated the liver to become more effective in thyroxine decomposition. Kot and Klitgaard (12) showed that rats fed desiccated thyroid to produce +70%

[2] Standard deviation.

BMR excreted nearly twice as much C^{14} labeled thyroxine in the bile, and 50% more in the urine than normal rats. Monroe and Turner (8) observed increased excretion of thyroxine in goats which were fed or injected with thyroxine, but they also stated that thyroxine destruction rather than excretion per se was the predominating factor in the elimination of excess thyroxine. O'Neal (5) showed that the speed of elimination of thyroxine increased as its level in the blood was increased. In the normal state thyroxine destruction and excretion has been found to balance secretion (13). When exogenous thyroxine is supplied in large amounts the regulating organs of the body try to eliminate the excess, so as to maintain a normal state (5, 8, 12).

In the cow which is constantly fed thyroxine as in this experiment it is evident that metabolic or gastrointestinal destructive processes for excess thyroxine are overwhelmed at the start of thyroxine feeding, but that they become more efficient with continued treatment. This is indicated by peak PBI levels at the earliest stages, in spite of the fact that in cows 23 and 51 greater amounts (up to twice as much) of thyroxine were fed later with a resulting lower PBI level. It was also evident that the level of circulating thyroxine was not controlled by excretion of thyroactive compounds. In fact, the thyroxine activity excreted followed the trend of the PBI changes in the blood. If PBI had been controlled through excretion per se, excretion should have increased as PBI declined at the higher dose levels. Since this did not occur, the major method of regulating excess body and gastrointestinal thyroxine must be the metabolic or gastrointestinal destruction and/or degradation of thyroxine to inactive compounds.

These observations appear to disagree with the results reported by Gross and Leblond (14) of excretion rates of I^{131} labeled L-thyroxine from rats. They found that as the dose was increased both the percentage of the I^{131} dose excreted and the proportion of thyroxine-like (butanol soluble) I^{131} increased. It is possible that the excretion and metabolism pattern after single doses as used by Gross and Leblond (14) would be considerably different than after continuous feeding as in this experiment. The butanol soluble I^{131} also may have included excretion compounds of low biological activity.

The large difference between cows 51 and 23 in PBI and heart rates along with smaller differences in rate of thyroxine equivalent excretion indicate that metabolic destruction of thyroxine was more effective in cow 23 than in cow 51. In addition to their age difference, cow 23 had been fed thyroxine in previous lactations. The possibility that the previous experience had already developed efficient mediums of destroying excess thyroxine in cow 23 should not be ignored. The apparently increased effectiveness of excess thyroxine destruction which occurred in all of these cows as thyroxine feeding was prolonged must be at least part of the homeostatic response which has been noted in lactating cows.

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THE INITIATION OF THYROID FUNCTION IN THE MOUSE[1]

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ABSTRACT

In the thyroid of BUB mouse fetuses of different ages the 24-hour I^{131} uptake and radiochromatographic distribution was studied concurrently with the histological appearance. The development of thyroid function in the mouse fetus takes place between 15 and 17 days of age. The ability to accumulate iodide from the circulation, the ability to bind this iodine organically, and the formation of colloid appear between 15 and 16 days of age; whereas the ability to produce thyroxine and the formation of follicles appear between 16 and 17 days of age. It is concluded that colloid formation is primary to follicle formation and that the production of thyroxine begins later than the collection and organic binding of iodine.

MANY studies have investigated when, during embryonic development, the thyroid begins to function. The first histological signs of thyroid secretion appear at comparatively different times in the gestation periods of various mammals. Similarly the times at which the successive steps in the synthesis of thyroid hormone appear, and the degree of histological differentiation of the thyroid tissue with which these steps coincide, vary among species (1–5). In the mouse the first uptake of iodine by the fetal thyroid has been reported at 15 and at 16 days of age, and the first appearance of colloid and follicles has been found to follow the first iodine uptake by one day (6, 7). No reference has been found for the first organic binding of iodine or for the first thyroxine formation in the mouse. In order to determine when in the mouse fetus the different aspects of thyroid hormone production appear and with which histological advancement these stages in metabolic activity coincide, the following approaches have been made:

1) Study of the histological appearance of thyroids of fetuses, 14 through 19 days of age;

2) Study of autoradiograph slides of thyroids of fetuses, 14 through 19 days of age, which had been injected with I^{131} 24 hours previously;

3) Determination of the 24 hour I^{131} uptake of thyroids of fetuses, 14 through 19 days of age; and

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4) Radiochromatographic analysis of thyroids of fetuses, 16 through 19 days of age, which had been injected with I^{131} 24 hours previously.

MATERIALS AND METHODS

Albino mice of the inbred strain BUB/Wi were maintained at 78° C, fed a low iodine diet and allowed water *ad libitum*. Pregnancies were dated by the vaginal plug method and the ages were recorded as described by Kauffman *et al.* (8). In this strain the gestation period is 19 to 20 days. Twenty-four hours after the injection of a carrier-free tracer dose of NaI^{131} a pregnant mouse was sacrificed and the fetuses were removed. The thyroid of each fetus was dissected out along with a piece of the trachea in order to avoid loss of radioactivity due to direct handling. For the histological studies the thyroids were fixed in Bouin's fluid and stained with hematoxylin and eosin or with periodic acid-Schiff and hematoxylin. For the autoradiograph studies the thyroids were likewise fixed in Bouin's fluid, and the slides were stained after, rather than before, the dipping and developing in order to prevent decolorization. The deparaffinized slides were dipped in NTB photographic emulsion[2] at 42° C in the darkroom with a Wratten #1 filtered light. After a suitable exposure period they were developed in Kodak D-19 in complete darkness, followed by fixation in Kodak acid fixer. The slides were completed by staining with hematoxylin and eosin.

For the 24-hour I^{131} uptake determinations each thyroid was placed in a vial with 1 cc. of 7N KOH and hydrolyzed in a boiling waterbath. The radioactivity was counted in a well-type scintillation counter. For the determination of body background a piece of liver and kidney of each fetus was counted. From each pregnant mother 4 fetuses were assayed, except in a few cases where the pooled thyroids of several fetuses were counted.

For the radiochromatographic analyses the thyroids of a number of fetuses of the same age were pooled, ground in a glass mortar and suspended in 1 cc. veronal buffer (pH 8.6), containing 7×10^{-4} M PTU and 4 mg. of trypsin. This was incubated for 3 days at 38° C. After $1\frac{1}{2}$ days of incubation an additional 4 mg. of trypsin was added. Extraction was carried out with 3 ml. of water-saturated n-butanol, followed by 3 further extractions with 1 ml. of solvent. The combined extract was evaporated at room temperature and dissolved in a 66% alkaline alcohol solution. One-dimensional, descending chromatograms were prepared on Whatman #1 filter paper with n-butanol saturated with 2N acetic acid as the solvent system. For identification the following carriers were run concomitantly: thyroxine, diiodotyrosine, monoiodotyrosine[3] and sodium iodide. The carriers were visualized by spraying with a 0.2% ninhydrin solution in n-butanol with 5% acetic acid and with a 1% palladium chloride solution. In order to make radioactivity distribution curves the sheets were separated into 3 cm. wide strips, which were passed by 0.5 cm. steps in front of a Geiger-Müller scanning counter. Each reading was recorded and plotted on graph paper. The curves showed separate peaks for: unhydrolyzed thyroglobulin, iodide, MIT, DIT, and thyroxine, the latter including triiodothyronine. The areas of the components were measured with a planimeter and from these the relative quantity of each compound was calculated.

RESULTS

The results of the chromatographic analyses are summarized in Table 1. Figure 1 represents the 24-hour I^{131} uptake by the thyroid at different

[2] The photographic emulsion was generously provided by Eastman Kodak Company.

[3] The following abbreviations have been used: MIT for monoiodotyrosine; DIT for diiodotyrosine.

TABLE 1. CHROMATOGRAPHIC DISTRIBUTION OF THE RADIOIODINATED AMINO ACIDS IN THE THYROID OF FETAL MICE OF DIFFERENT AGES 24 HOURS AFTER I^{131} INJECTION

Age of fetus in days	Number of samples	Percent glandular radioiodinated compounds*				
		Thyroxine	DIT	MIT	I^-	Unhydrolyzed thyroglobulin
16	2	—	23.9	13.3	50.4	12.4
17	4	46.4	19.6	17.8	11.7	4.5
18	6	51.3	16.8	20.8	7.8	3.3
19	4	45.8	18.0	23.6	9.1	3.5
adult†	3	35.6	14.3	35.3	11.5	3.3

* The values represent the percentage of the radioactivity on the chromatograms attributable to each compound.

† The adult values were obtained from female mice treated similarly to the fetus-bearing females.

prenatal ages. Photomicrographs of fetal thyroid of different ages are shown in Figures 2 through 5. From the results obtained by the different techniques the following pictures evolve for each of the prenatal days studied:

In the 14-day-old fetus the thyroid tissue consists of irregular cords of cells and there is no uptake of iodine.

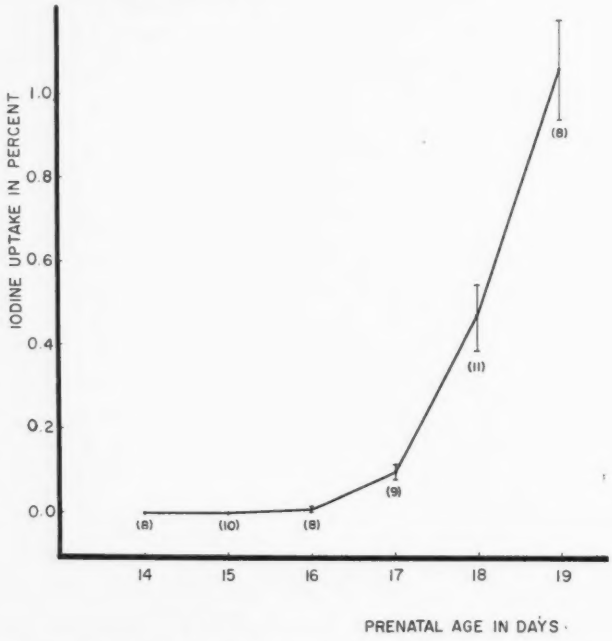


FIG. 1. The uptake of iodine by the fetal mouse thyroid in relation to prenatal age. The iodine uptake is expressed as the percent of the dose injected 24 hours previously. The standard error of the mean is represented by vertical lines. The number of litters examined for each point is indicated in parentheses.

The thyroid tissue of the 15-day-old fetus still consists of irregular cords of cells and does not differ essentially from the previous day. There is no uptake of iodine; even when 4 or more fetal thyroids were pooled in one sample, the counting rate did not exceed the background.

The difference between the thyroid of the 16-day fetal thyroid and that of the previous day is striking. The tissue is interlaced with blood vessels. There are no follicles, but among the cells small triangular or star-shaped droplets of colloid can be seen (Fig. 2). At 16 days the fetal thyroid is able to collect iodide from the circulation. The percentage of iodine taken up is actually small, but amply significant in all 8 litters studied. Chromatographic analysis of 16-day fetal thyroid shows that the iodine taken up is partially in the inorganic form, but some is bound organically as MIT and DIT (Table 1). Since the total I^{131} uptake is so small at 16 days, the preponderance of DIT over MIT is not necessarily significant. No thyroxine could be detected in the chromatograms of the 16-day fetal thyroid. Autoradiograph slides of this same day show some activity above the cells, but most silver grains are clustered above the small droplets of colloid among the cells, indicating that the organic iodine is concentrated mostly in the small intercellular colloid droplets.

In the thyroid of the 17-day-old fetus the first follicles are present. In addition, colloid droplets can be seen in and among the cells not yet arranged in follicles (Fig. 3). The thyroid takes up a considerable fraction of the injected radioiodine. This iodine is converted to MIT and DIT, as well as to thyroxine. Again, the autoradiograph slides show that the organic iodine is concentrated mostly in the colloid.

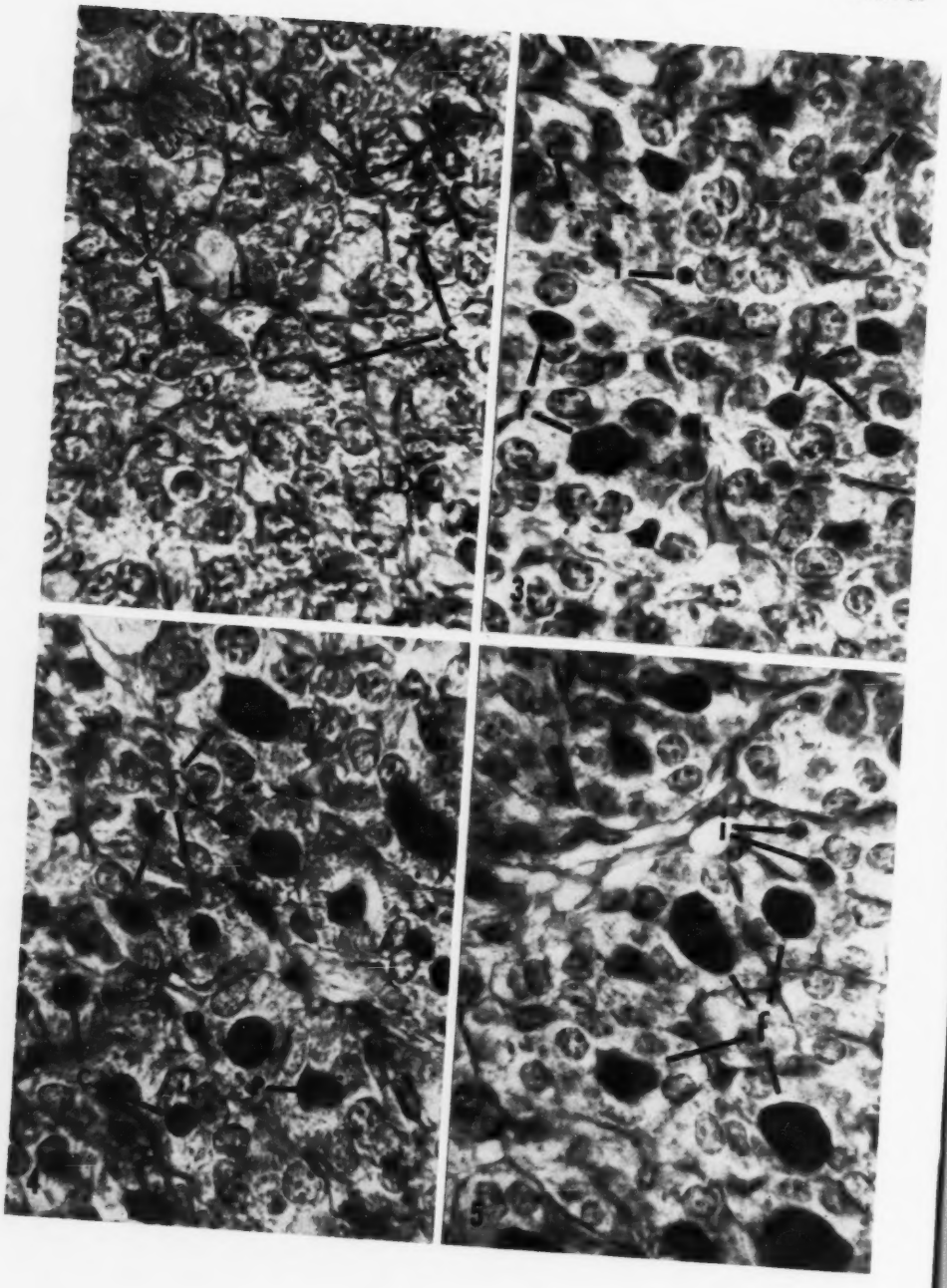
The thyroid tissue of the 18-day-old fetus is not essentially different from the previous day; colloid is found in follicles and also in and among cells (Fig. 4). An increased amount of iodine is taken up by the thyroid and is bound organically as MIT, DIT and thyroxine in approximately the same proportion as at 17 days.

The difference between the 19-day-old fetal thyroid and the previous one is only quantitative; the follicles are somewhat larger in size and the nonfollicular colloid droplets are somewhat scarcer (Fig. 5). The iodine uptake has again increased. The radiochromatographic distribution of the iodine resembles that of the 17- and 18-day-old fetal thyroid.

For comparison the chromatographic distribution of the 24-hour iodine uptake of adult thyroids is included in Table 1. The adult thyroid differs from that of the 17-, 18- and 19-day-old fetal thyroid in that it contains less thyroxine and more MIT. The high ratio of MIT to DIT probably reflects the low iodine content of the diet, which these mice received (9).

DISCUSSION

The initiation of thyroid function in the mouse fetus occurs between 15 and 17 days of age. In the 15-day-old fetal thyroid no iodine is taken up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and the epithelium consists only of irregular cords of cells. In the 17-day-old mouse fetus the iodine which is taken up by the thyroid is incorporated into MIT, DIT and thyroxine, and colloid-filled follicles are present. The finding that no qualitative changes either in chromatographic distribution or in histological appearance of the thyroid were found on later days implies that complete thyroid function has begun at 17 days of age. The results of the intermediate day, 16 days of age, show the manner of development of thyroid function: the small amount of iodine taken up is partially bound organically to MIT and DIT, but not yet to thyroxine, and colloid is present in small spaces, but not yet in follicles. Therefore, the ability to accumulate iodide from the circulation, the ability to bind this iodine organically to MIT and DIT, and the ability to produce colloid develop between 15 and 16 days of age; whereas the ability to synthesize thyroxine and the ability to form follicles develop between 16 and 17 days of age. These results are in agreement with the finding of Speert *et al.* (7) that the fetal mouse thyroid first accumulates radioiodine after 16 days of gestation. Using the same strain (Swiss mice), but pooling several thyroids from one litter, Jacobson and Brent (6) found that already in the 15-day-old fetal thyroid selective iodine accumulation takes place. In the present investigation, when the thyroids of the younger fetuses were pooled, no significant uptake in the 15-day fetal thyroid could be detected.

Since in the biosynthesis of thyroid hormone in the adult animal iodide is first concentrated from the circulation, then combined with tyrosine to form MIT, further combined to DIT, and finally to thyroxine, it might be expected that in the fetal thyroid the ability to collect iodide is present before the ability to bind iodine organically, that MIT is present before DIT and that DIT in turn is present before thyroxine. This indeed has been found in the chick, but not in other species. Trunnell and Wade (1) found in the chick the first selective iodine collection by the thyroid in the 5-day-old embryo, the first organic binding to MIT at $8\frac{1}{2}$ days, to DIT at $9\frac{1}{4}$ days and the first formation of thyroxine at $9\frac{3}{4}$ days; after 10 days no more qualitative changes were observed. On the other hand, in the rabbit, according to Waterman and Gorbman (3) iodide concentration begins at

FIG. 2. Thyroid tissue of a 16-day-old mouse fetus. Note the presence of small, irregularly shaped colloid droplets (c) and the abundance of blood vessels (b). PAS ×1000.

FIG. 3. Thyroid tissue of a 17-day-old mouse fetus. Note the presence of small follicles (f), as well as colloid droplets which are not located in follicles, but within (i) and among (c) cells. PAS×900.

FIG. 4. Thyroid tissue of an 18-day-old mouse fetus. In addition to follicles (f), colloid droplets are still present within (i) and among (c) cells. PAS×900.

FIG. 5. Thyroid tissue of a 19-day-old mouse fetus. The follicles (f) are somewhat larger, and occasionally nonfollicular colloid droplets (i) can still be seen. PAS×900.

15 days of age and at 17 days MIT and DIT as well as thyroxine, are present in the fetal thyroid. In the mouse, as found in the present investigation, the accumulation of iodide and the organic binding to MIT and DIT appear simultaneously, while the formation of thyroxine appears the following day. Therefore, the complete development of thyroid function in the mouse and the rabbit takes two to three days, whereas in the chick it takes six days. This longer developmental period plus the use of shorter assay intervals might possibly explain why a stepwise appearance of the stages in thyroid hormone synthesis was found in the chick. A stepwise development of the stages in thyroid hormone synthesis in the mouse and the rabbit is not excluded; the steps may follow in rapid succession and thus escape detection.

Since in the adult thyroid the organic binding of iodine takes place in the colloid (10), it might be expected that in the fetal thyroid colloid would be present when the first organic binding of iodine occurs. This seems to be the case in some, but not in other species. In the fetal calf Koneff *et al.* (4) found thyroxine present a number of days prior to the formation of colloid droplets and follicles. A similar situation was met in the pig (11). On the other hand, in the chick, Wollman and Zwilling (2) found small PAS-positive colloid droplets in the fetal thyroid on the same day as the first organic binding of iodine. In the rat (5) and the rabbit (3) colloid-filled follicles were found to appear at the time of the first iodine binding. Similarly, in the mice of the present experiments the first organically bound iodine and the first traces of colloid appeared on the same day.

The slides of the fetal mouse thyroid at different ages imply that colloid formation is primary to follicle formation. The 16-day fetal thyroid contains small droplets of colloid among the cells, but no follicles are present. The colloid droplets increase in size, become surrounded by epithelial cells and form the follicles as seen in the 17-day fetus. On later days more new follicles are formed seemingly by intra- and intercellular colloid droplets increasing in size and becoming surrounded by epithelial cells. The secretion of colloid is therefore concluded to precede the formation of follicles. In some investigations follicle formation is presented as primary to colloid production and this concept is illustrated by pictures which show empty follicles and follicles slightly filled with colloid (rabbit, 3; mouse, 7). In the present investigation empty follicles without colloid were not observed. On the other hand, the present description of follicle formation agrees with Sugiyama's finding in rats and mice, that the follicle cavity is formed by expanding secretion into the intercellular fissures (12). Experiments in which fetal thyroid is explanted and cultured *in vitro* also show colloid droplets appearing in advance of follicles (13, 14).

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PREPARATION AND PROPERTIES OF THE COFACTOR OF AN ACTH-INACTIVATING ENZYME SYSTEM FROM HUMAN BLOOD[1]

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ABSTRACT

The isolation from human blood of a cofactor of an *in vitro* enzyme system inactivating ACTH is described. The cofactor accelerated the loss of activity of ACTH as measured by the Sayers and Sayers bioassay in the presence of either a blood protein fraction (Cohn III-O) or plasminogen. The ACTH-inactivating activity of the latter in the present system does not appear to be identical with its fibrinolytic action.

THE rapid inactivation of ACTH[4] in heparinized plasma of rat, rabbit and man was reported by Reiss *et al.* (1). Geschwind and Li (2) found that sheep ACTH preparations were readily inactivated when incubated with rat liver and kidney slices, diaphragm, and homogenates of liver, kidney and adrenal tissues, but that they were inactivated only partially by boiled tissues; the supernatant fraction of centrifuged liver homogenate possessed most of the inactivating potency. In the experiments of Geschwind and Li incubation of ACTH with rat blood plasma did not result in loss of activity. White and Gross (3) reported that the splitting by bovine fibrinolysin of corticotropin-A resulted in loss of biological activity. Mirsky *et al.* (4) observed that ACTH is susceptible to hydrolysis by streptokinase-activated plasma or plasminogen. Pincus, Hopkins and Hechter (5) described an ACTH-inactivating system in mammalian blood. This consisted of an enzyme fraction obtained from bovine, human or rat blood and a dialyzable cofactor present in heat-inactivated human and rat blood. The present paper is a report on further studies in which attempts were made to concentrate and purify the cofactor. It was also found that plasminogen can be substituted for the crude enzyme preparation previ-

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[4] Abbreviations used: ACTH, adrenocorticotropin; EDTA, ethylene diamine tetraacetic acid.

ously used and that it inactivates ACTH in the presence of a highly purified cofactor, the preparation of which is described.

EXPERIMENTAL AND RESULTS

Materials

The ACTH was a corticotropin of porcine origin purified on oxycellulose (lot No. 101900), generously supplied by Dr. C. E. Graham, Wilson Laboratories, Chicago. Its activity was: subcutaneously, 102 U.S.P. units per mg.; intravenously, 40 U.S.P. units per mg.

Cohn (6) fraction III-O (prepared from bovine plasma), plasmin and plasminogen were supplied by Dr. W. F. White of Armour Laboratories, Chicago.

Assay of Enzyme and Cofactor Activity

The ACTH was dissolved in 0.1N HCl and diluted with Krebs phosphate saline, pH 7.2 (7) containing 0.002 M EDTA. The latter was added to prevent the spontaneous inactivation of ACTH which occurred during incubation with the saline medium. This solution was adjusted to contain 0.8 I.U. of corticotropin per ml. The enzyme and cofactor preparations were dissolved in 1.4 ml. of phosphate saline; 0.1 ml. of the corticotropin solution was added and the mixture was incubated for one hour in a Dubnoff shaking incubator at 37°. The reaction mixture was then diluted with saline to a volume of 10 ml. and 1 ml. portions were injected into the femoral veins of each of five hypophysectomized male rats (80–100 grams) for the determination of the residual ACTH activity by Munson's modification (8) of the Sayers and Sayers adrenal ascorbic acid depletion method. Under the conditions described, 50–75% of the ACTH was inactivated. Although 100% inactivation could be observed, this was avoided as undesirable because of the uncertainty of stopping the reaction at the precise moment this point was reached.

Concentration of Cofactor Activity

Outdated human citrated blood plasma was obtained from the local blood bank and was stored in the frozen state. For each preparation, one or two liters of plasma were allowed to melt, diluted with one-half volume of water and immediately deproteinized by running into the mechanically stirred solution an excess of 3N perchloric acid. The coagulated protein was separated by centrifugation and was re-extracted with water to which perchloric acid had been added. The combined extracts were filtered through Whatman No. 1 filter paper. The clear filtrate was neutralized by adding solid K_2CO_3 with stirring until the pH was just above 7, and left in the refrigerator overnight. The precipitated $KClO_4$ was removed by filtration and the clear solution was stirred for 30 minutes with activated charcoal (Nuchar C-190 granular) which was then removed by filtration.

At this stage the solution did not show any cloudiness when tested with perchloric or trichloroacetic acid, but gave a copious precipitate with 5% phosphotungstic acid in 2N HCl.

The filtrate after the charcoal treatment was now transferred to a large flask and at least 5 volumes of cold acetone were added. On standing in the cold room for several days a white precipitate formed. The aqueous acetone was removed by decantation and the solid material resuspended in fresh cold acetone and dried by repeatedly centrifuging and resuspending in acetone with grinding in a mortar. It was finally collected on a Buchner filter and dried in a desiccator under vacuum. A part of the material which adhered as a fine film to the wall of the vessel in which it had been precipitated was dissolved in a small volume of water and the solution was lyophilized. The lyophilized material was combined with the acetone-dried material. Yield: about 3 gm. of dry powder from 1 liter of plasma. A 10% aqueous solution of this material was found to have cofactor activity in the bioassay. The solution could be stored in the frozen state for several months without apparent loss of activity.

Fractionation of the Cofactor-active Material

Fractionation of the crude material was at first attempted by various methods including ion-exchange chromatography using the resins Dowex 50 and Amberlite IRC 50 (XE 64). Paper electrophoresis, which afforded a more convenient and clear-cut separation, was adopted as the method of choice for the preparation of a small amount of highly purified active material. A Spinco electrophoresis cell (Durrum type) was used, a whole sheet of filter paper (Whatman No. 1 washed with methanol and water) replacing the customary eight narrow strips. The buffer was 0.1 M ammonium acetate, pH 6.8; 0.1 ml. of a 10% solution of the crude powder was applied as a thin line along the top of the sheet. With a voltage of 150 v., which was required to maintain a current of 20 ma., separation was achieved in three hours. The paper was dried at room temperature and three distinct zones were located by spraying guide strips with ninhydrin. Of these the largest fraction had remained at the point of origin. Two smaller fractions had migrated toward the cathode and anode respectively. Only the immobile zone was stained by Naphthylamine Black (9).

When 20 sheets had accumulated the zones were cut out and separately eluted with 0.002 M NH_4OH. The eluates were filtered and lyophilized. Both the cationic and the anionic fractions were again dissolved in water and subjected to electrophoresis. Each fraction yielded in addition to the main constituent a small amount of the fraction of opposite charge. The purified fractions thus obtained are designated as "re-run" or "twice-run" material. On further testing by electrophoresis no further cross-contamination was detected.

TABLE 1. ENZYMATIC INACTIVATION OF ACTH

Enzyme	Cofactor	No. of assays	Inactivation of ACTH (%)		
			Range	Mean	Standard deviation
Fraction III-O	—	7	—	—	—
Fraction III-O	plasma	6	40–68	51	9.5
Plasminogen	—	8	—	—	—
Plasminogen	crude powder	12	56–80	74	13.3

The incubation mixture contained: 80 mU. corticotropin, either human blood protein fraction III-O, 26 mg., or bovine plasminogen, 1 mg. The cofactor was either deproteinized human plasma equivalent to 1 ml. of blood plasma or a solution of 1 mg. of crude cofactor powder. The total volume was 1.5 ml. Incubation period 60 min. Temperature 37°. The activity of the corticotropin in the reaction mixture was determined by bioassay (8).

Biological Activity of the Fractions

Approximately 1 ml. of deproteinized blood plasma was required to activate 26 mg. of Cohn fraction III-O so as to produce 50% inactivation of 80 mU. of ACTH. In this system 26 mg. of fraction III-O could be replaced by 1 mg. plasminogen. The crude cofactor powder, 1 mg. of which corresponded to ca. 0.3 ml. of the original blood plasma, caused 64–85% inactivation of ACTH when added in a concentration of 0.67 mg. per ml. (Table 1). The purest ("twice-run") cathodic fraction was active as cofactor when present in the incubation mixture in a concentration of 1 μg per ml. (Table 2). Thus the cofactor activity of the crude powder precipitated from blood plasma had been concentrated at least 500 fold by the purification procedure described. The "twice-run" anodic fraction and the immobile fraction did not show activity in comparable dilutions.

Inactivation of ACTH by Plasmin

Unlike plasminogen, plasmin inactivated ACTH without the need to add cofactors. Its activity was abolished by the addition of antifibrinolysin. Some samples of blood plasma fraction III-O which had been stored for more than one year had acquired ACTH-inactivating activity not requiring activation by the cofactor. This activity was also abolished by antifibrinolysin (Table 3).

TABLE 2. INACTIVATION OF ACTH BY PLASMINOGEN AND PURIFIED COFACTOR

Cofactor (μg.)	Inactivation of ACTH (%)
0	0
1	30
8	40
40	48

Experimental conditions as in Table 1. The enzyme was 1 mg. plasminogen; the cofactor was a "twice-run" cathodic fraction.

TABLE 3. EFFECT OF ANTIFIBRINOLYSIN ON ACTH-INACTIVATING SYSTEMS

Enzyme	Amount in incubation mixture (mg.)	No. of assays	Inactivation of ACTH (%)		
			Range	Mean	Standard deviation
Plasmin	0.05	7	50–78	69	8.6
Plasmin Antifibrinolysin	0.05 0.20	4	0–25	13	3.3
Fraction III-O (aged)	2.60	10	64–90	78	8.8
Fraction III-O (aged) Antifibrinolysin	2.60 0.20	4	0	0	

The initial amount of ACTH in 1.4 ml. incubation mixture was 80 mμ.

Fibrinolytic Activity of Plasmin and Plasminogen

In view of the well-known fibrinolytic activity of plasmin (10), it might be expected that the addition of the cofactor resulted in the activation of plasminogen to plasmin. However, this was not the case since the cofactor was ineffective in causing fibrinolysis when added to plasminogen in the fibrin plate test (11).[5]

Properties of Cofactor-Active Material

Both the cathodic and the anodic material were, owing to the volatility of the buffer employed, free from salt. They were buff powders, hygroscopic when exposed to the atmosphere, but stable when stored in a vacuum desiccator at room temperature. They were not soluble in organic solvents except in formamide.

Apart from the ninhydrin reaction given by the three zones and the staining with Naphthylamine Black of the immobile zone, it was difficult to detect other color reactions. Since only small amounts of the purified material were available for this purpose, it is possible that some reactions might be observable if larger quantities were available. The anthrone reagent of Roe (12) revealed the presence of carbohydrate in the immobile zone. The color reaction was intense, whereas corresponding amounts of the mobile zones did not show any color.

Infrared analysis (KBr pellet) of the purest cathodic material revealed the presence of carbonyl groups, amino groups (and possibly OH groups), but no bands suggesting the presence of phosphorus.

Molecular weight determinations in the ultracentrifuge[6] indicated a molecular weight of the purified material of the order of magnitude of 1500.

The mobility of the cathodic material in the Tiselius moving boundary electrophoresis apparatus (Perkin Elmer) in ammonium acetate buffer

[5] We wish to thank Dr. Perkins P. K. Chang for this information.

[6] We wish to thank Dr. G. Kegeles for permission to use his ultracentrifuge and for help and instruction in its operation.

pH 5.35 was 2.97×10^{-8} cm.^{2} sec.$^{-1}$ volt^{-1} for the descending peak and 2.89×10^{-8} cm.^{2} sec.$^{-1}$ volt^{-1} for the ascending peak.

Paper Chromatography and End Group Analysis of Cofactor

The "twice-run" cathodic and anodic zones ran on paper as single spots which showed different mobilities. The solvent system tertiary butanol:water:formic acid 70:15:15 (v/v/v) allowed faster movement and better separation of the crude material into its components than n-butanol:acetic acid:water (13). In both solvents the cathodic material had greater mobility than the anodic zone.

The dinitrophenyl derivative (14) of the cathodic material was heated with 6N HCl in a Carius tube at 110° for 48 hours. The hydrolysate was extracted with ether and both the ether extract and the aqueous fraction evaporated. On filter paper impregnated with phthalate buffer pH 6.0 (15) the dinitrophenyl derivative of the unhydrolyzed material moved more slowly than the ether extract of the product of hydrolysis, which had a mobility comparable to that of the DNP derivative of tryptophan. The aqueous portion of the hydrolysate, however, moved as slowly as the unhydrolyzed derivative and may have contained unhydrolyzed material.

Ultraviolet Absorption Spectra

Aqueous solutions of both the purified cathodic and anodic fractions showed visible fluorescence in ultraviolet light. The absorption spectra in ultraviolet light of dilute solutions (40–200 μg per ml.) of both zones showed an inflection point at ca. 275 mμ. The inflection point was shifted slightly in alkaline solution and after acid hydrolysis.

The presence of tryptophan is suggested by the results of the end group analysis and by the inflection point in the ultraviolet spectrum; its shift on acid hydrolysis may be due to the destruction of tryptophan.

Photometric determination of amino groups according to Moore and Stein (16) showed that 1 mg. of the purest fractions gave a ninhydrin color corresponding to the color produced by 12–15 μmoles of leucine which was used as a standard.

From the relationship between the absorption in ultraviolet light of the solution and its ninhydrin color, a "molecular" absorption coefficient, based on leucine, was calculated for the terminal amino groups of the cofactor. The purest anodic fraction had $\epsilon_{275}(\text{leucine}) = 0.654 \times 10^4$. The purest cathodic fraction had $\epsilon_{275}(\text{leucine}) = 0.560 \times 10^4$. This coefficient was useful in the determination of the concentration of solutions of small amounts of material which, owing to its hygroscopic nature, could not be weighed accurately.

DISCUSSION

Whereas the ACTH-inactivating system described by other workers (1, 2, 3, 4) consisted of blood, animal tissues or tissue extracts, the present

study shows that the highly purified system used in this study contained two components, an enzyme-protein which may be related to or identical with plasminogen and a non-protein cofactor required for the activation of the enzyme.

It is notable that the purified cofactor employed in the present system is a heat-stable, dialyzable substance of relatively low molecular weight (less than 2000), itself devoid of activity. The cofactor is therefore not related to streptokinase, which was employed by Mirsky *et al.* (4) to activate blood plasma or plasminogen. It differs from other known activators of plasminogen which with two exceptions are proteolytic enzymes (10).

Since no evidence of activation of the fibrinolytic activity of plasminogen by the cofactor has been obtained, it is probable that the enzymatic mechanism of fibrinolysis by plasmin differs from its mechanism of ACTH inactivation.

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RELATION OF THE HYPOTHALAMUS TO ANTERIOR PITUITARY THYROTROPIN SECRETION[1]

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ABSTRACT

Methods are described by which thermal lesions were made in rat hypothalami and their placement rapidly confirmed histologically.

Bilateral thermal lesions placed in the anterior ventral hypothalamus of 100 gm. female Wistar rats resulted in a significant and permanent depression of thyroid secretion rate as measured by the rate of loss of previously assimilated I^{131}. This depression was effective less than 6 hours after lesions had been made.

Rats with thyroid-depressing lesions retained a significant depression of both 24-hour uptake of I^{131} and rate of release of incorporated I^{131} from their thyroids when measured 2, 4, and 8 weeks post operatively.

Rats with thyroid-depressing lesions required only 1.0 μg Na *l*-thyroxine /100 gm./day to prevent thyroid secretion (loss of incorporated I^{131}) in the presence of 5–10 mg. methylthiouracil daily, while unoperated control rats required 3.0–4.0 μg daily.

It was concluded that the depression of the secretion rate after making the lesions described was the result of an increased sensitivity of the thyrotropin secreting cells of the anterior pituitary to the action of thyroxine entering the systemic circulation.

LESIONS in the anterior hypothalamus of the rat may depress thyroid function by causing a depression of thyrotropin secretion (1, 2, 3, 4). Lesions of this kind moderate, but do not prevent, the response of the pituitary to thyroxine deficiency (5).

The experiments reported here were commenced in an attempt to examine the function of the hypothalamus which might modify anterior pituitary thyroid stimulating activity.

MATERIALS AND METHODS

Experimental Animals

Female Wistar rats from the University of Otago Medical School colony were used throughout these studies. Operations were performed when the rats reached 98–102 gm., the stereotaxic instrument being modified for rats of this size.

Stereotaxic Instrument

The stereotaxic instrument used was modified from the design of Krieg (6) and resembled that described by Greer, Menecken and Bolen (7). In our instrument the top of

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the palate bar was at the same height as the axis of the ear plugs. The ear plugs were shaped specially to obtain greater accuracy in the lateral placement and to avoid damage to the internal ear (i.e. faults of conical ear plugs). The special shaping is shown in Figure 1. The plug A has a diameter turned to fit the auditory meatus of rats the size to be operated on (i.e. 100 gm.). Lateral placement is ensured by the pressure of the step B on the edge of the bony meatus, and so does not depend for accuracy on both meati being of the same diameter.

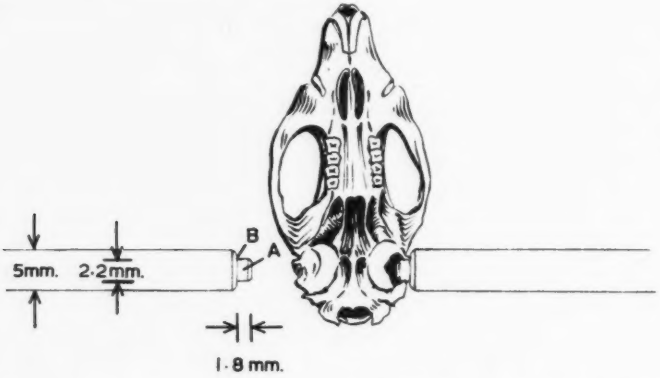


FIG. 1. Design and dimensions of ear plugs used in stereotaxic instrument for rats of 100 gm. (see text for description).

Electrodes

The electrodes were made from sewing needles soldered into hypodermic needle hubs, and were insulated with coil varnish. The tips were subsequently ground to form a conical tip with an included angle of 60°. A Luer-lok boss, fastened to the stereotaxic instrument but insulated from it by bushings of perspex, was used to hold the electrodes.

Alternating current at low voltage was used at radiofrequencies, delivered from a device similar to that described by Kaufer (8), the lesions thus being due to thermal destruction. For the lesions described below 14.5 m.a. at 13.0 volts was passed for 20 seconds. No current was passed through the electrodes in sham operated controls.

Placement of Lesion

In all rats lesions were made bilaterally with the tips of the electrode placed 5.8 mm. anterior to the ear bars, 1.0 mm. to right and left of the mid-line, and 1.0 mm. from the floor of the cranial cavity. The vertical co-ordinate for the floor of the cranial cavity was previously determined in 6 rats and measured in the mid-line at the same anterior co-ordinate.

At the conclusion of each study all operated rats were killed with coal gas, and their brains, thyroids, adrenals, ovaries and uteri removed and fixed in 10% formalin. Pituitaries were removed, and fixed and sectioned as previously described (9).

Endocrine organs were weighed after 1 week in fixative. The hypothalami were dissected from the fixed brains, using a sharp razor blade. A horizontal cut was made through the tissues above the corpus callosum, and the block containing the hypothalamus roughly trimmed square. To indicate the left side of the hypothalamus in the sections the anterior left corner was cut from the square. The hypothalami were then mounted in 10% gelatine and refixed in formalin until sectioned.

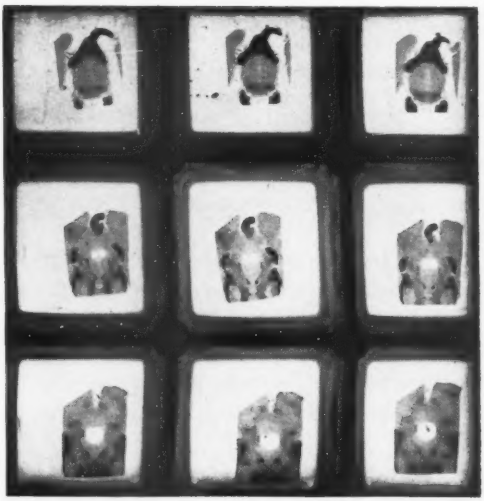


FIG. 2. Sections 26–28, 40–42, 54–56 from 90 serial sections of hypothalamus of rat bearing thyroid-depressing lesions. Lesions do not extend into sections 26–28. Seen in staining fluid in subdivided plastic container. (Transmitted light, ×2.)

Serial 40 μ sections were cut in the horizontal plane on a freezing microtome commencing from the ventral surface. Individual sections were placed in consecutive chambers of subdivided clear plastic trays, each chamber containing 1 cc. of staining fluid (see Fig. 2).

The staining fluid was 0.05% toluidine blue containing 50 mg. sodium nitrite/600 cc. to prevent corrosive oxidation of the microtome knife.

Each section was stained for 16 hours, 1 drop (about 0.05 cc.) of acetic acid buffer solution of pH = 3.2 being added to each chamber to prevent overstaining. The acetic acid buffer contained 2 gm. sodium acetate and 10 cc. glacial acetic acid in 100 cc. of water.

The trays containing the stained sections were examined, using transmitted light and an optical magnifier (×20), and every 10th section mounted. From these sections the diameter of the lesions and their placement with respect to various hypothalamic structures were determined, using an ocular micrometer.

Diet

A pelleted diet with low but variable I^{127} content was fed to rats in Experiment I. A standard low iodine diet containing 72% maize meal, 16% wheat gluten, 10% brewers yeast, 1% $CaCO_3$, 1% NaCl, was fed throughout Experiments II and III to ensure uniform high thyroid uptakes of injected I^{131}.

Measurement of thyroid responses

The effectiveness of the lesions in altering thyroid activity was determined by measurement of the uptake or the rate of release of radioactive iodine (I^{131}) in the thyroids. *In vivo* neck counts were made over a scintillation counter shielded with a lead plate 1" thick through a window 1 cm. square. At least 2,000 counts were recorded for each animal, the counting error being approximately ±2% for each count.

Uptake was calculated from the neck counts at 24 hours after injection of I^{131} and expressed as a percentage of the dose injected.

Rate of release was found by calculating the percentage of the thyroid I^{131} content at any time which left the gland over a 24-hour period.

RESULTS

A. *Effects of lesions on thyroid function*

Experiment I:

Bilateral thermal lesions were made in the anterior ventral hypothalamus in 3 groups of rats. Two, four, and eight weeks later, each group, with its litter mate controls, was given 2 μc I^{131} and the neck counts recorded at 24, 48, 72, and 144 hours after injection. The results of these counts are shown in Table 1.

TABLE 1. DURATION OF THE SUPPRESSIVE EFFECTS OF HYPOTHALAMIC LESIONS ON THYROID I^{131} UPTAKE AND RELEASE IN RATS

Time since lesion operation (weeks)	No. of rats	Type	Mean % I^{131} in thyroid at 24 hr. (with S.E.)	Mean loss I^{131} from thyroid (%/day) (with S.E.)
2	5	Lesions	24.6 ± 1.51*	5.2 ± 1.05**
	3	Controls	61.7 ± 4.77	17.5 ± 1.02
4	3	Lesions	17.2 ± 0.85*	9.1 ± 3.50*
	5	Controls	37.5 ± 2.38	37.7 ± 2.29
8	3	Lesions	33.7 ± 2.58*	4.3 ± 0.59*
	2	Controls	86.8 ± 0.71	28.6 ± 0.75

Values of P for differences between rats with lesions and their controls:
* $0.001 < p < 0.01$ ** $0.01 < p < 0.05$

Both uptake and subsequent rate of release of I^{131} from thyroids were significantly lower for rats with lesions than for their controls. This effect was observed as early as 2, and as late as 8, weeks post operatively.

Experiment II:

To test how soon, post operatively, the thyroid-depressing effect of the lesions became established, 21 rats were given 3 μc I^{131} when approximately 95 gm. in weight, while on the standard low iodine diet. From 24 hours later to the end of the experiment each rat was kept on the standard low iodine diet and injected twice daily with 5 mg. methylthiouracil (MTU) and given 0.05% MTU in the drinking water.

Neck counts were made to establish the rate of release of I^{131} from the thyroids, and those rats which had reached 98–102 gm. were subjected to bilateral lesions as described above. Thyroid counts were made 6 hours later and then twice daily thereafter.

From Table 2 it can be seen that in the 24-hour period following the

TABLE 2. RATES OF LOSS OF I^{131} FROM THYROIDS OF RATS BEFORE AND AFTER MAKING HYPOTHALAMIC LESIONS

	No. of rats	Mean rates of loss I^{131} from thyroids (%/24 hr. with S.E.)		P value
		Before operation	After operation	
Lesions made	7	49.6 ± 3.93	17.2 ± 2.99	<0.001
Sham Operation	14	50.9 ± 3.46	58.8 ± 3.28	N.S.

All rats received 5 mg. MTU 2×daily (I/P) and 0.05 % MTU in drinking water throughout the experiment.

lesion operation the rate of release of I^{131} was significantly reduced in the rats with lesions when compared (a) with their controls, and (b) with their own pre-operative rates of release. Figure 3 demonstrates this effect and it is evident that the rate of thyroid release of I^{131} was already depressed by the 6th hour after placement of lesions and continued at the reduced level thereafter for the 3 days over which it was followed. By comparison the sham-operated control rats maintained rapid release of I^{131} from their thyroids.

As under the MTU regime imposed on these rats, the rate of release of I^{131} from the thyroids reflects the rate of pituitary TSH secretion, it was concluded that TSH secretion was reduced less than 6 hours after placement of hypothalamic lesions.

Experiment III:

An attempt was made to obtain a quantitative measure of the sensitivity of pituitary TSH secretion to the depressant action of thyroxine after hypothalamic lesions. Twenty-two rats were subjected to bilateral lesions at the co-ordinates previously employed. After 14 days on the standard low iodine diet, to ensure measurable I^{131} uptakes, all rats, to-

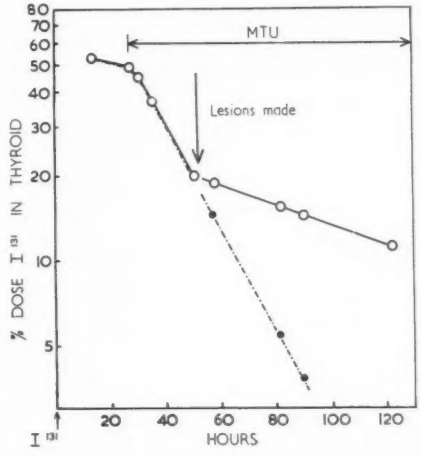
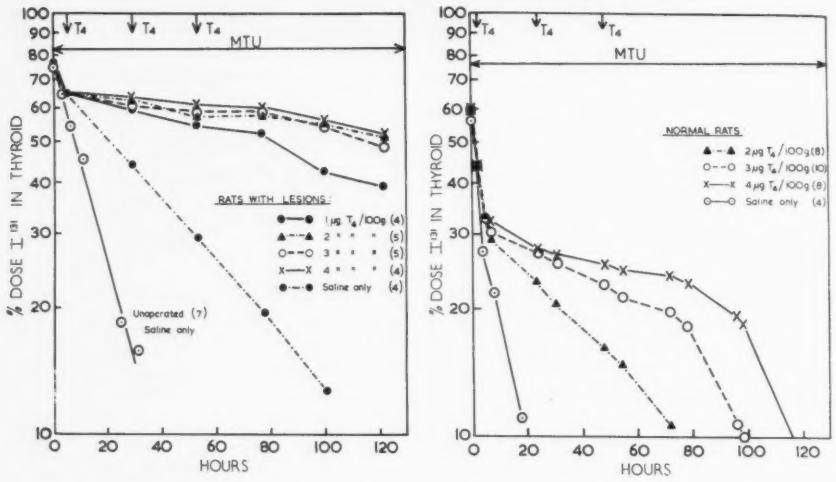


FIG. 3. Alteration of thyroid I^{131} release rate by hypothalamic lesions in rats.
○——○ rats with lesions.
●·—·—· ● control rats.
MTU = Methyl thiouracil 0.05% in drinking water and 5 mg./rat 2× daily (I/P).



(a) (b)

FIG. 4 (a and b). Sensitivity to endogenous thyroxine of rats with hypothalamic lesions and unoperated controls.

T4 = injection of Na *l*-thyroxine

MTU = Methyl thiouracil 0.05% in drinking water and 5 mg./rat/day injected.

No. of animals in parentheses.

gether with 7 unoperated controls, were injected intraperitoneally with 3 μc I^{131}. Fourteen hours later thyroid counts were commenced. Each rat was injected daily with 5 mg. MTU, given drinking water containing 0.05% MTU, and fed the standard low iodine diet until counting was terminated.

The 22 rats with lesions were divided into 5 equivalent groups. Groups 1–4 received 4.0, 3.0, 2.0 and 1.0 μg Na *l*-thyroxine/100 gm. live weight in 0.5 cc. saline daily. Group 5 and the unoperated controls both received saline (0.5 cc.) only.

Experiment III was then repeated using unoperated control does. Thirty does were divided into 4 groups. Groups 1–3 were injected daily with 4.0, 3.0 and 2.0 Na *l*-thyroxine/100 gm. in 0.5 cc. saline, and group 4 received 0.5 cc. saline daily.

Thyroxine injections were made subcutaneously and were given for 3 days. *In vivo* counts continued for 6 days. The results of these experiments are shown in Figure 4.

Daily levels of thyroxine above 1.0 μg/100 gm. were equally effective in preventing release of I^{131} from thyroids of rats with lesions. Loss of I^{131} from thyroids of rats receiving 1.0 μg thyroxine/100 gm. daily was also markedly depressed compared with the saline injected lesion con-

trols. When the daily thyroxine injections stopped the group of rats which had received 1.0 μg/100 gm./day was the first to resume thyroid secretion. Unoperated control rats lost I^{131} from their thyroids at a faster rate than the rats with lesions when both groups received saline only.

In the trial using unoperated rats, both 3.0 and 4.0 μg thyroxine daily suppressed I^{131} release from the thyroids, while 2.0 μg daily had only a partial effect. When the thyroxine injections ceased, does which had received 3.0 μg/100 gm. daily resumed thyroid secretion before those which had received 4.0 μg.

B. *Other effects of the lesions*

Of 43 rats in which lesions were made 3 died within 1–3 days of operation, due to refusal to eat. One rat exhibited hyperphagia, which resulted in obesity, the remainder growing at normal rates post operatively. Six of the operated animals exhibited some degree of ferocity when handled.

Examination of daily vaginal smears of 31 does with lesions revealed that while 18 had normal cyclic oestrous patterns, 12 exhibited the phenomenon of persistent vaginal cornification, and 1 of absence of cornified cells from daily smears. Of 17 does with normal smear patterns which mated, 10 littered and 3 died at full term. None of the 10 which littered produced sufficient milk to prevent death of their young within 3–5 days post partum.

C. *Areas of hypothalamus destroyed by lesions*

In all rats the lesion holes appeared circular in horizontal section. Destruction was first apparent about 0.2 mm. above the ventral surface of the optic tracts and continued above this point in an average number of 65 sections each of 40 μ thickness. In the 12 most dorsal and 12 most ventral sections, the diameters of the holes were increasing towards the included 40 sections in which lesions were of maximal diameter = 1.5 ±0.1 mm. (mean from 21 rats). Each lesion hole thus extended 1.6 mm. at maximal diameter with terminal cones of destruction extending for about 0.5 mm. both dorsally and ventrally.

Anteriorly, the lesions often impinged on the posterior margin of the optic tracts and the suprachiasmatic nuclei. Posteriorly, lesions extended into the dorsomedial nuclei and laterally they destroyed the fornices and medial portions of the lateral hypothalamic areas and median forebrain bundle. The lesions coalesced in the mid line and destroyed the paraventricular nuclei and structures immediately ventral to them, including the anterior extremities of the ventromedial nuclei. The lesions did not extend into the median eminence.

D. *Pituitaries*

Pituitaries from does with lesions were found to have considerably fewer stainable thyrotroph cells than did pituitaries of control does. The neural lobes were never atrophic and all contained stainable neurosecretion.

DISCUSSION

Damage from electrolytic lesions is diffuse and variable, both in form and extent (10), and Clark (11) was unable to correlate symptoms with areas of hypothalamic damage after electrolysis. Thermal lesions as made in these experiments improve on electrolytic lesions by being more precise in form. Electrolytic lesions made in the anterior hypothalamus have been reported to extend into the median eminence (4) and adenohypophysis itself (1). No such ramifications of destruction followed placement of thermal lesions, the destroyed areas being cylindrical in form about the lesion electrode and circular in horizontal section. Vertically, the lesions were centered on the upper margin of the uninsulated tip of the electrode, showing that some heating damage had occurred above the insulated extremity of the needle. A similar observation has been made by Morrison *et al.* (10).

Hypothalamic obesity, aphagia and ferocity occurred here in relatively few of the 43 does subjected to bilateral thermal lesions in contrast to the incidence of aphagia and death (1) and of hyperphagia (4) following electrolytic lesions. It is believed that the smallness of the proportion of does so affected in these experiments was due to the more circumscribed nature of the damage which results from thermal lesions.

Examination of serial sections of the hypothalami of all operated animals is essential to any study which attempts to ascribe functions to specific hypothalamic areas or nuclei. By the methods described above, it was found that the hypothalamic structures completely destroyed in these rats included the paraventricular nuclei and sections of the fornices at the same dorsal level. Destruction of tissues also occurred in the anterior hypothalamic areas and in the medial extremities of the lateral areas. Little damage was sustained by the suprachiasmatic or ventromedial nuclei and no damage occurred in the median eminence (see Fig. 5). The area destroyed was thus comparable to that affected in other studies concerned with thyroid activity (12, 13). Smaller thermal lesions made medially, which destroy the paraventricular nuclei and structures immediately ventral to them, reduce thyroid activity to the same extent as the large lesions above (14).

Experiment I above confirms earlier reports showing that both uptake and release of I^{131} from the thyroids of rats with lesions in the anterior hypothalamus depressed 12–14 days post operatively (3, 4). Experiment I extends these observations by demonstrating that the depression of thyroid activity was still present eight weeks after operation. Subsequent studies

have shown that the thyroid activity remained depressed in rats 40 weeks after operation, and it may be said that this effect of such lesions is permanent.

By making lesions in animals which were releasing I^{131} from their thyroids, with re-uptake blocked by MTU in Experiment II, it was found that thyroid release rate (effectively a measure of pituitary thyrotropin (TSH) secretion rate under these conditions) was depressed within less than six hours of making the lesions.

As the reduced rate of loss of I^{131} from the thyroids of rats which received lesions was detected at *in vivo* counts made six hours after operation, the

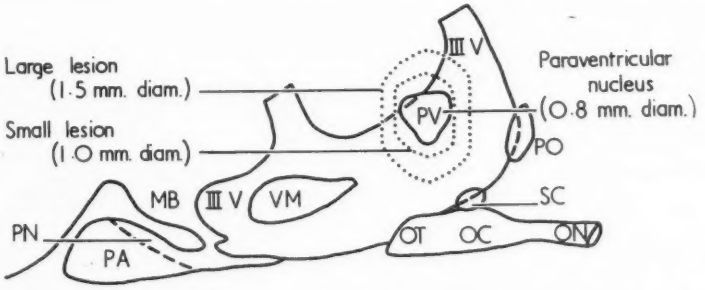


FIG. 5. Diagrammatic representation of hypothalamus of a rat showing the position of the large lesion described above and the smaller lesion of Averill and Purves (14), both of which depress thyroid function.

MB = mammillary area	OC = optic chiasma
PA = anterior pituitary	PN = posterior pituitary
PO = preoptic area	SC = suprachiasmatic nuclei
IIIV = third ventricle	VM = ventromedian nuclei

depression of TSH release must have been effective some time before this count was made. It is in fact detectable as early as four hours after placing lesions, but as the anaesthetic and operational procedures themselves usually cause a small temporary depression in thyroid I^{131} release rate, it may not be possible to determine, by this method, precisely how soon the function of the pituitary is affected after damaging the hypothalamus.

Injection of small amounts of thyroxine into the pituitary glands of rabbits (15) and rats (16) cause an almost immediate reduction in TSH secretion rate, showing that the pituitary itself is sensitive to small local increases in thyroxine concentration. Pituitaries grafted to sites other than the median eminence of rabbits (17), of guinea pigs (18) and of mice (19, 20), are sensitive to injected thyroxine. From both types of experiment it is clear that the pituitary itself is sensitive to thyroxine levels.

Experiment III above showed that in rats bearing thyroid-depressing lesions, 1.0 μg Na *l*-thyroxine per 100 gm./day was sufficient to suppress pituitary TSH secretion. In normal rats 3.0–4.0 μg/100 gm./day was

necessary to suppress pituitary TSH secretion, a figure similar to that reported earlier (21). It was concluded that destruction of tissues in the ventral anterior hypothalamus had rendered the anterior lobe of the pituitary about three times more sensitive to injected thyroxine.

In what way the lesions have operated to produce this change is not yet clear and must be elucidated by further experiment. It may be that the sensitivity of the anterior lobe tissue itself to thyroxine is not altered by the lesions, but that after the lesions have been made more of the injected thyroxine reaches the anterior pituitary lobe. This is possible because the anterior lobe is supplied with blood which has first passed through the capillaries of the median eminence and is then distributed to the anterior lobe by the hypophysial portal system. Brown-Grant (22) and Purves (23) have suggested that the median eminence normally removes or inactivates significant amounts of the thyroid hormone in the blood passing through it and that the extent of this removal or inactivation is controlled by the hypothalamus. The effect of the lesions may be to diminish the extent of the removal or inactivation of thyroid hormone in the median eminence, and thus make the anterior lobe more sensitive to the thyroid hormone in the systemic circulation. While this explanation seems to us the most probable one, it is admitted that with the evidence at present available other explanations are possible.

It is important to note that the "screening" or "filter" theory of median eminence action, as outlined above, assumes that the sensitivity of the anterior lobe cells to the thyroid hormone which reaches them is not altered by hypothalamic lesions. Furthermore, injections of thyroid hormone given once a day are not a suitable way to test the screening action because of the probability that the high hormone levels produced in the blood for a short time after each injection will swamp the screening action. This may be the reason why neither D'Angelo (4) nor we have been able to demonstrate an increased sensitivity of the anterior lobe to triiodothyronine injected once a day in rats with thyroid-depressing hypothalamic lesions.

Florsheim (24) has reported experiments similar to ours in which he, unlike us, did not find a differential sensitivity to thyroxine injected once a day after hypothalamic lesions. Two features of Florsheim's experiments may have contributed to this result. Firstly, his thyroxine injections were given intramuscularly and would have been absorbed more rapidly than from the subcutaneous site used by us. Secondly, his experiments were performed in the summer, at a time when the thyroxine requirement of the control animals was already unusually low (Florsheim, personal communication).

Acknowledgments

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PLASMA CATECHOL AMINE LEVELS AND ADRENAL ASCORBIC ACID CONTENT FOLLOWING β-MERCAPTOETHYLAMINE (MEA) ADMINISTRATION

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ABSTRACT

The effect of β-mercaptoethylamine (MEA) on the peripheral plasma catechol amine level and the adrenal ascorbic acid content of rats was studied. Peripheral plasma catechol amine concentrations were determined at 10, 30 and 60 minutes following intraperitoneal injections of 125 mg./kg. of MEA. An increase of plasma epinephrine concentration occurred 10 minutes after the administration of MEA and this elevation persisted throughout the one hour observation period. Similar results were obtained after the administration of cystamine, the dimer form of MEA.

The I.P. administration of 125 mg./kg. of MEA to intact rats caused a significant decrease in adrenal ascorbic acid content at the end of one hour as compared to saline control rats. However, MEA did not have any effect on the concentration of adrenal ascorbic acid of hypophysectomized rats. These results indicate that the decrease of adrenal ascorbic acid of intact rats following MEA treatment is not due to a direct action of the chemical on the adrenal gland as suggested by some investigators, but by action through the hypophysis.

IT HAS been shown that β-mercaptoethylamine (MEA) produces a fall in the liver glycogen of mice (1). Sokal *et al.* observed a hyperglycemia and a marked depletion of liver and muscle glycogen of the rat following the administration of MEA. Ergotamine pre-treatment or adrenal demedullation prevented the hyperglycemia and muscle glycogenolysis. The authors suggested that MEA stimulates the secretion of epinephrine (2). A recent study revealed that the administration of MEA, in large radioprotectant doses to dogs, produced a rise in circulating epinephrine accompanied by a hyperglycemia (3).

It has been suggested that epinephrine may stimulate ACTH secretion, resulting in an adrenal ascorbic acid depletion of the rat (4, 5). Van Cauwenberge and co-workers observed that MEA caused a fall in adrenal ascorbic acid of the rat, but concluded that this chemical did not act by releasing ACTH (6). However, the latter group of investigators used only intact animals.

The purpose of this study is two-fold, first, to determine whether there

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is a rise in plasma catechol amines following the administration of MEA and secondly, to assess the role of the hypophysis in MEA-induced adrenal ascorbic acid depletion using intact and hypophysectomized rats.

EXPERIMENTAL METHODS

Catechol amines and blood glucose

Walter Reed rats of either sex which had been fasted for 18–24 hours were used. A dose of 125 mg./kg. of MEA (base) or 100 mg./kg. of the dimer form of MEA (base), 2,2,dithio-bis-(ethylamine), cystamine, was administered intraperitoneally. The chemicals were given as isotonic solutions and as the hydrochloride salt. The animals were anesthetized with 35 mg./kg., I.P., of pentobarbital 10 minutes before the blood samples were taken. Estimates of plasma catechol amines were made by means of a modification (7) of the Weil-Malherbe and Bone method (8) on 3 ml. of blood taken from the abdominal aorta by means of a needle puncture following laparotomy. One ml. of blood taken either from the inferior vena cava or abdominal aorta from another group of rats was analyzed for blood glucose by the method of Nelson (9). All the blood samples were taken from the different groups of rats before, and at 10, 30, and 60 minutes after drug administration.

Ascorbic acid determinations

The Munson modification (10) of the Sayers, Sayers and Woodbury (11) technique for assaying ACTH activity was used as a basis for the design of this experiment. Charles River male rats weighing from 203 to 260 gm. were fed and allowed water *ad libitum*. The animals were divided into four groups. Group I consisted of hypophysectomized rats which received intraperitoneal injections of 125 mg./kg. of MEA (base). Group II were hypophysectomized rats which received equal volumes of 0.9% sodium chloride solution intraperitoneally. Group III contained intact rats receiving MEA (125 mg./kg. base, I.P.) while the rats in Group IV served as their saline controls. The hypophysectomized rats had undergone surgery approximately 40 hours before the start of the experiment. One hour after the administration of MEA or saline the animals were anesthetized with sodium pentobarbital (6 mg./100 gm. of body weight, I.P.). The adrenal glands were removed, weighed and placed in 6% trichloroacetic acid solution within 3 minutes. The glands were ground and the ascorbic acid determinations were performed on the filtrates according to the method of Roe and Kuether (12).

At the end of the experiment, the sellae of the hypophysectomized rats were examined carefully, by means of a dissecting microscope, for completeness of hypophysectomy.

RESULTS

Plasma catechol amines

The plasma epinephrine level of the 10 minute post MEA treated rats was estimated to be 15.4 μg/liter as compared to 0.7 μg/liter in the saline control group ($P < .001$). This elevation was more marked 30 minutes after MEA treatment (24.6 μg/liter). One hour after MEA administration, the plasma level of epinephrine was observed to be 22.7 μg/liter ($P < .001$). The dimer form of MEA, cystamine, produced even greater rises in plasma epinephrine. The plasma level was estimated to be 33.4 μg/liter at 10 minutes after cystamine treatment, compared to a control level of 0.7 μg/liter ($P < .001$). At 30 minutes the plasma concentration of epinephrine

TABLE 1. CATECHOL AMINE PLASMA LEVELS FOLLOWING THE ADMINISTRATION OF MEA OR CYSTAMINE

Treatment	Epinephrine μg/liter				Norepinephrine μg/liter			
	Mean ±	S.E.	n	P	Mean ±	S.E.	n	P
Saline	0.7	0.19	11		4.0	1.16	11	
10 Minutes Post MEA	15.4	1.30	6	<.001	0.8	0.85	6	>.10
30 Minutes Post MEA	24.6	4.91	6	<.001	7.6	5.01	6	>.05
60 Minutes Post MEA	22.7	3.85	6	<.001	11.2	6.45	6	>.05
10 Minutes Post Cystamine	33.4	2.69	6	<.001	8.2	1.79	6	>.05
30 Mintues Post Cystamine	35.4	3.92	5	<.001	11.9	2.35	5	<.02
60 Minutes Post Cystamine	47.1	6.67	6	<.001	12.9	4.98	6	>.05

had risen to 35.4 μg/liter and at the end of 60 minutes the level was 47.1 μg/liter. The plasma norepinephrine level rose from 4.0 to 11.2 μg/liter in the MEA group and to 12.9 μg/liter in the cystamine group one hour after the respective chemical was given (Table 1).

Blood glucose values

The blood glucose levels (Fig. 1) rose from 94.3 ± 2.2 (Mean ± S.E.) mg. percent to 104.9 ± 7.7 mg.% 10 minutes after MEA was given. At 30 minutes the blood glucose level (118.4 ± 4.1) was elevated over the saline control group ($P < .001$). The hyperglycemia persisted for the next 90 minutes. Cystamine caused an even greater elevation of the blood glucose than MEA. The glucose level rose from 94.8 ± 5.3 mg.% to 128.0 ± 4.3 mg.% at the end of 1 hour ($P < .001$).

Adrenal ascorbic acid content

The mean adrenal ascorbic acid values (Table 2) of the saline control rats was 396.0 μg/100 mg. of adrenal tissue while the adrenal ascorbic acid concentration of the animals receiving MEA fell to 262.9 μg/100 mg. of adrenal tissue ($P < .001$). However, the adrenal ascorbic acid level of the hypophysectomized rats treated with MEA was almost identical to their hypophysectomized saline treated controls (465.0 and 464.1 μg/100 mg. of adrenal tissue, respectively).

DISCUSSION

The present data show that there is an epinephrine increase in the plasma of the rat following MEA administration; plasma epinephrine concentra-

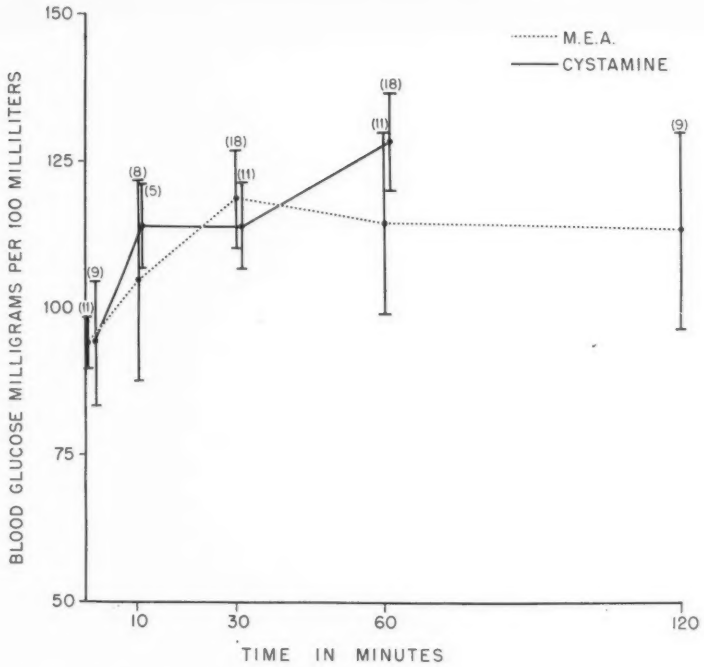

FIG. 1. Blood glucose levels following the administration of 125 mg./kg. of MEA or 100 mg./kg. cystamine. Bars indicate ±2 S.E. from the mean. Numbers in ( ) = number of experimental animals.

tion of dogs has also been shown to increase under similar circumstances (3). This substantiates Sokal and co-workers' suggestion that MEA may stimulate the release of epinephrine in the rat (2). The plasma catechol amine level rose to quite a large titer and this elevation persisted through-

TABLE 2. ADRENAL ASCORBIC ACID CONTENT OF INTACT AND HYPOPHYSECTOMIZED RATS TREATED WITH 125 MG./KG. BETA-MERCAPTOETHYLAMINE

Treatment	Micrograms ascorbic acid /100 mg. tissue		n	t	p
	Mean	±S.E.			
Group I-MEA Hypophysectomized	465.0	20.34	12		
Group II-Saline Hypophysectomized	464.1	25.06	12		
Group III-MEA Intact Control	262.9	16.18	10	4.44	<.001
Group IV-Saline Intact Control	396.0	25.09	12		

out the one hour observation period. The hyperglycemia which was observed seemed to parallel this rise. In other studies, using MEA treated rats, liver glycogenolysis was not observed until 30 minutes after an I.P. injection (2). It was at this period that we obtained the first significant rise in blood glucose levels. Sokal *et al.* reported that adrenal demedullation prevented the hyperglycemia which normally followed MEA treatment in the rat. These investigators also demonstrated that MEA could cause liver glycogenolysis in adrenal demedullated and the depancreatized rat indicating that this agent may have an effect independent of the adrenal medulla or glucagon action. However, muscle glycogenolysis did not occur after adrenal medullectomy (2). So it appears from the above evidence that the hyperglycemia that is observed after MEA treatment is due to epinephrine release.

It has long since been suggested that epinephrine causes a release of ACTH from the pituitary of the rat (4). Bacq and Alexander have stated that adrenal ascorbic acid depletion alone may be insufficient as evidence for the assertion that stress is present (13). However, adrenal ascorbic acid depletion has been widely accepted as an index of ACTH activity and has, in fact, been demonstrated to be an excellent assay for this hormone under proper conditions in hypophysectomized rats (10, 11, 14). It is not known how much of the adrenal ascorbic acid depletion, following the administration of MEA, might have been mediated through the release of epinephrine in these experiments. It is suggested that under the conditions of our experiments it could have played a role. Experiments are now in progress to study this factor by use of adrenal demedullated rats.

Although Van Cauwenberge *et al.* observed an adrenal ascorbic acid depletion (27% in one hour and 65% in 4 hours) after the administration of MEA, they concluded that this chemical does not act to stimulate ACTH secretion. These investigators arrived at this thought because they were unable to demonstrate a significant decrease of cholesterol concentration in the adrenal gland (26% at 4 hours) or a significant decline of circulating eosinophiles (30% at 5 hours) of rats given MEA (6). However, this does not rule out the possibility that ACTH may be released. Perhaps MEA alters the physiology of the rat to such a degree that the production of cortical hormone and its action on leucocytes is modified. In any event, the fact remains that hypophysectomy prevents the depletion of ascorbic acid from the adrenal gland of the rat treated with MEA. Therefore, we think that this depletion is due to a stimulation of ACTH secretion from the anterior pituitary, which may be brought about by release of epinephrine, probably acting in some manner through the hypothalamus (15).

Acknowledgment

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STUDIES ON THE METABOLISM OF ADIPOSE TISSUE: THE EFFECT OF ADRENALINE ON OXYGEN CONSUMPTION AND GLUCOSE UTILIZATION[1,2]

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ABSTRACT

The effect of adrenaline on the oxygen consumption of adipose tissue *in vitro* has been studied. The oxygen uptake of rat epididymal adipose tissue is linear with time and is independent of the presence of glucose in the medium. Adrenaline, 10 μg per ml., inhibits the oxygen uptake of adipose tissue incubated in a protein-free medium whether glucose is present or not. When albumin is present in the medium, adrenaline scarcely alters the oxygen uptake in the absence of glucose but stimulates oxygen uptake in the presence of glucose. The inhibition of oxygen uptake by adrenaline is related to the accumulation of free fatty acids within the tissue; the presence of albumin in the medium reduces the intracellular concentration of free fatty acids and prevents the inhibition of oxygen uptake. In the presence of insulin adrenaline markedly stimulates oxygen uptake; glucose is required for this effect. Insulin by itself scarcely alters oxygen uptake.

IN PREVIOUS studies in this series (1) the action of adrenaline, alone and in combination with insulin, on the net gas exchange and glucose utilization of adipose tissue was reported. Adrenaline appeared to divert the metabolic pathway of glucose, taken up under the influence of insulin, away from fatty acid synthesis to other metabolic products such as lactic acid. The present study is concerned with the effect of adrenaline on the oxygen consumption of adipose tissue, and the relationship of the response to free fatty acid liberation. The influence of glucose, insulin, and serum albumin upon this action of adrenaline is also reported.

METHODS

Male white rats (170–220 gm.) obtained from the Holtzman Company and maintained on a diet of Purina laboratory chow *ad libitum* were used in all experiments. The procedure for the housing of the rats and the removal and handling of the tissue was as previously described (2) (3). Only two pieces of tissue were used from each rat, namely, those referred to in previous studies as the distal portions (3).

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[2] This is the sixth in a series of reports devoted to studies on the metabolism of adipose tissue.

Tissues were incubated in Warburg vessels with a total volume of 16–20 ml. After a preliminary temperature equilibration of 15 minutes at 37°, oxygen consumption was measured for up to 3 hours. The incubation medium (2.8 ml.) was either a phosphate buffered inorganic salt solution (4) containing half the recommended concentration of calcium, or a glycylglycine-buffered salt solution which had the following composition: 76 ml. 0.154 M NaCl, 4 ml. 0.154 M KCl, 3 ml. 0.11 M $CaCl_2$, 1 ml. 0.154 M KH_2PO_4, 1 ml. 0.154 M $MgSO_4$, 3 ml. 0.154 M $NaHCO_3$, 21 ml. 0.154 M glycylglycine buffer pH 7.4, 21 ml. water. A glycylglycine buffered medium was used in preference to the phosphate buffered medium since the full concentration of calcium could be used without the formation of a precipitate during the incubation. Similar results were obtained whether a phosphate-buffered or a glycylglycine-buffered medium was used. However, it was noted that the glycylglycine-buffered medium became more alkaline during incubation with intact adipose tissue reaching pH values of 7.8–7.9 after 2–3 hours' incubation. Paper chromatography of the medium showed that glycine was present in appreciable amounts. The nature of the enzyme in adipose tissue which is responsible for the cleavage of glycylglycine is at present unknown. In some experiments albumin (human serum albumin, fraction VR, from the Protein Foundation, Jamaica Plain, Massachusetts, or bovine serum albumin, fraction V, from Mann Biochemicals) was dissolved in the incubation medium to yield a 5% solution, and the solution adjusted to pH 7.4 with sodium hydroxide solution. The medium containing bovine serum albumin was dialysed against phosphate-buffered Krebs' solution before use. The albumin-containing glycylglycine-buffered medium showed negligible pH changes during incubation with tissue.

Glucose was added to the medium from the side-bulb (0.2 ml. of glucose solution in 0.9% NaCl) or was present in the main compartment from the start (replacing 0.2 ml. of the medium). The final concentration of glucose ranged from 90 to 360 mg. per 100 ml. Adrenaline and insulin solutions were prepared as described previously (2) (3). In preliminary experiments adrenaline blanks were run, i.e., the flasks contained all the components of the experimental flasks including adrenaline but the tissue was replaced by boiled tissue; no oxygen uptake was observed under these conditions. The center well contained 0.2 ml. of 20% KOH and a piece of rolled filter paper.

Oxygen uptake is calculated as μl. oxygen per 100 mg. of tissue (wet weight) per hour from the periods stated. The effect of adrenaline on oxygen uptake is measured by comparing one of a pair of tissues incubated with adrenaline with its paired tissue incubated without adrenaline and is expressed either as a percentage change, taking the control tissue oxygen uptake as 100%, or as an absolute change in μmoles oxygen.

Glucose was estimated according to the method of Nelson (5) as described previously (1). Lactic acid was estimated according to the method of Barker and Summerson (6). Free fatty acids (FFA[3]) were measured by the method of Dole (7); 0.5 ml. samples of the incubation medium were used. The tissues were homogenized in the acid extraction mixture (7) and made up to a volume of 10 ml.; 3 ml. of this homogenate were used for the FFA estimations. All estimations were done in duplicate.

RESULTS

The oxygen consumption of epididymal adipose tissue proceeds nearly linearly for as long as it has been followed (5 hours). Typical experiments are shown by the control curves of Figures 2 and 3. This rate of oxygen uptake is independent of the presence of glucose. In 5 experiments in which paired tissues were incubated, one with glucose, 360 mg. per 100 ml., and

[3] Abbreviation used: FFA, free fatty acids.

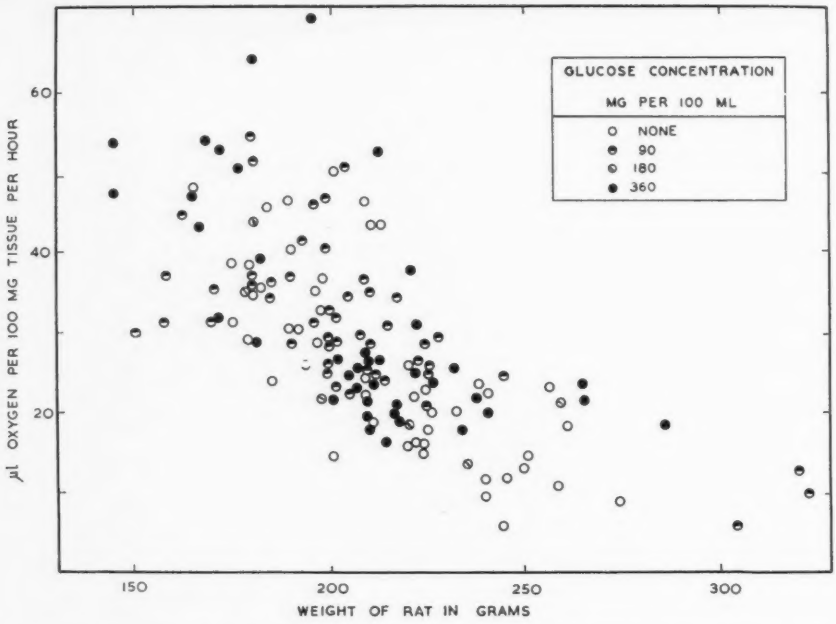

FIG. 1. Variation of the oxygen consumption of rat epididymal adipose tissue with the size of the rat.

The incubation medium was either glycylglycine-buffered or phosphate-buffered Ringer's solution containing the amount of glucose indicated. Values are calculated from the oxygen uptake during the first hour of incubation. Rats in the weight range 150–300 gm. are approximately 40–65 days old.

the other without, the mean oxygen uptakes were 28.1 and 27.7 μl. oxygen per 100 mg. tissue per hour respectively. The rate of oxygen consumption does depend on the age of the rat (Fig. 1). It is clear (a) that the rate of oxygen consumption decreases as the rat becomes larger, as has been observed previously (8) (9), and, (b) that there is considerable variation in the oxygen consumption from one rat to another within the same age group.

The principal effect of adrenaline on oxygen uptake of adipose tissue incubated in a protein-free medium is an inhibition of oxygen uptake; this is usually preceded by a brief stimulation of oxygen uptake (Fig. 2, A). In the presence of glucose the initial stimulation is greater but a similar inhibition is observed (Fig. 2, B). In 30 out of 36 rats such an inhibition was observed, but in six rats adrenaline stimulated oxygen uptake and the stimulation was maintained for 3 hours or more; in two of these 6 experiments no glucose was present in the medium. A possible explanation of the results obtained with these 6 rats might be the presence of glycogen in the tissue in greater than the usual concentration.

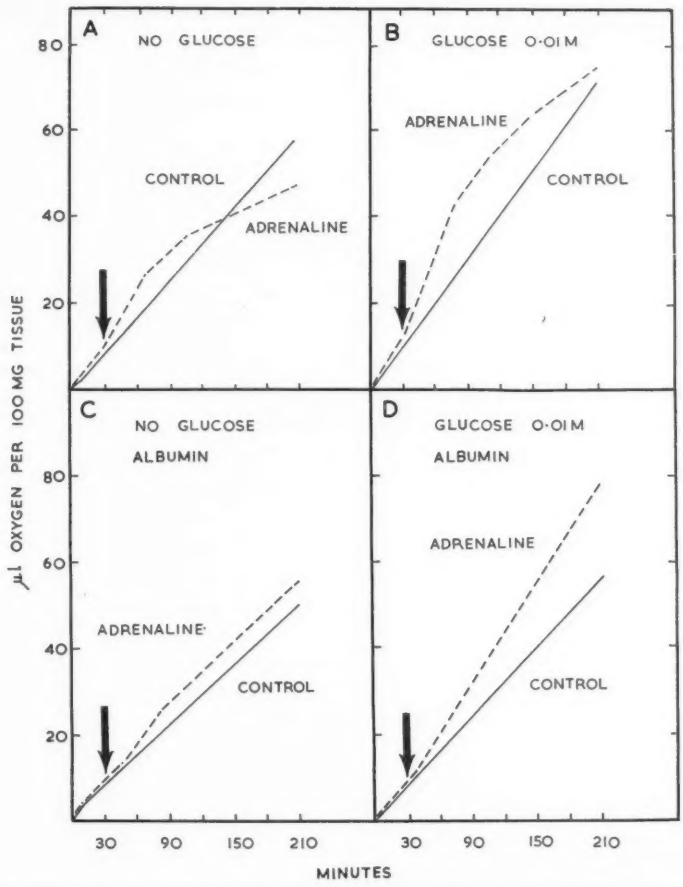

FIG. 2. Effect of adrenaline on the oxygen consumption of rat epididymal adipose tissue in the presence and absence of albumin and glucose.

The incubation medium was phosphate-buffered Kreb's solution. In experiments C and D the medium contained 5% bovine serum albumin (dialyzed). In experiments B and D the medium contained glucose, 180 mg. per 100 ml. Paired tissues were used in each experiment. After 30 minutes incubation adrenaline, final concentration 10 μg-per ml., was added to one of the tissues and 0.9% sodium chloride solution was added to the other tissue as indicated by the arrow. The weights of the rats were: A, 200 gm.; B, 200 gm.; C, 218 gm.; D, 222 gm. Readings in each case were made at ten minute intervals.

When albumin is present in the incubation medium adrenaline does not inhibit oxygen uptake (Fig. 2, C) and when glucose and albumin are present adrenaline stimulates oxygen uptake for up to 3 hours (Fig. 2, D). The inhibition of oxygen uptake by adrenaline observed in the absence of albumin appears to be related to the high concentration of FFA in the tissue (Fig. 3).

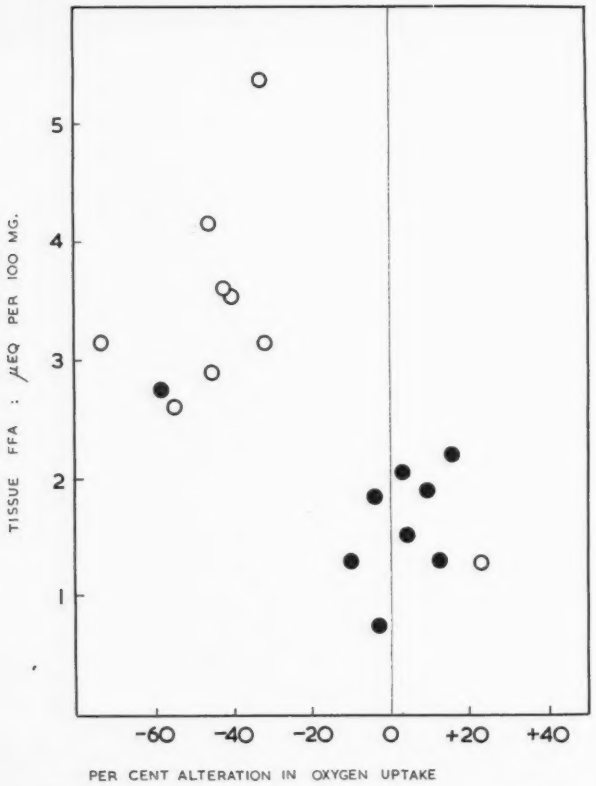

FIG. 3. Relationship between increased tissue FFA concentration and the inhibition of oxygen uptake caused by adrenaline.

The incubation medium was phosphate-buffered Ringer's solution. Experiments in which no albumin was present in the incubation medium are shown by open circles. Experiments in which albumin, 5 percent, was present in the medium are shown by solid circles. The percent alteration in the oxygen uptake caused by adrenaline is calculated from the oxygen uptake during the third hour after the addition of adrenaline, i.e., period 150′ to 210′ in Figures 2 and 3, and the oxygen uptake of the control during this same period; it is plotted against the FFA concentration in the tissue (μEq per 100 mg. tissue) at the end of the 3 hours' incubation with adrenaline.

When insulin and glucose are present in the incubation medium adrenaline markedly increases oxygen uptake (Fig. 4, B). This stimulation is not observed in the absence of glucose (Fig. 4, A). The stimulation of oxygen uptake by adrenaline in the presence of insulin and glucose is much greater than the stimulation in the presence of albumin and glucose.

In Figure 5 is shown the effect of adrenaline on oxygen uptake, glucose uptake and lactic acid production by tissue incubated in a protein-free medium. Under these conditions adrenaline did not markedly alter glu-

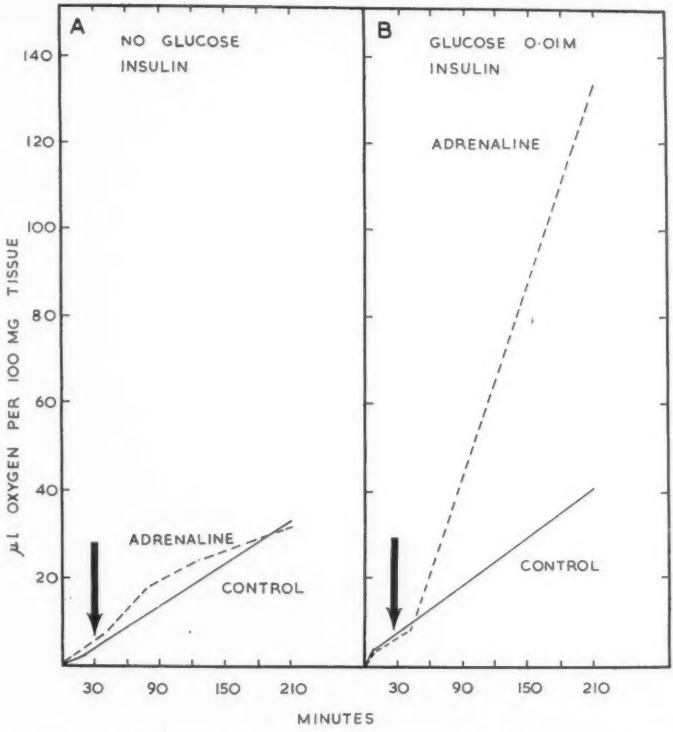

FIG. 4. Effect of adrenaline on the oxygen consumption of rat epididymal adipose tissue in the presence and absence of insulin and glucose.

The incubation medium was phosphate-buffered Kreb's solution containing insulin, 0.1 units per ml. In experiment B the medium contained glucose, 180 mg. per 100 ml. Paired tissues were used in each experiment. After 30 minutes incubation adrenaline, final concentration 10 μg per ml., was added to one of the tissues and 0.9 percent sodium chloride solution was added to the other tissue as indicated by the arrows. The weights of the rats were: A, 246 gm.; B, 246 gm. Readings were made at ten minute intervals.

cose uptake, decreased oxygen uptake and increased lactic acid production. Insulin by itself increased glucose uptake and lactic acid production and scarcely altered oxygen uptake. Insulin and adrenaline together increased glucose uptake to a slightly greater extent than did insulin alone, more than doubled the oxygen uptake and increased lactic acid production to a slightly greater extent than did insulin alone. Similar experiments in which albumin was present in the incubation medium show that adrenaline increases oxygen uptake and glucose uptake under these conditions; otherwise the results were very similar to those obtained in the protein-free medium.

Of the substrates other than glucose which have been used (pyruvate,

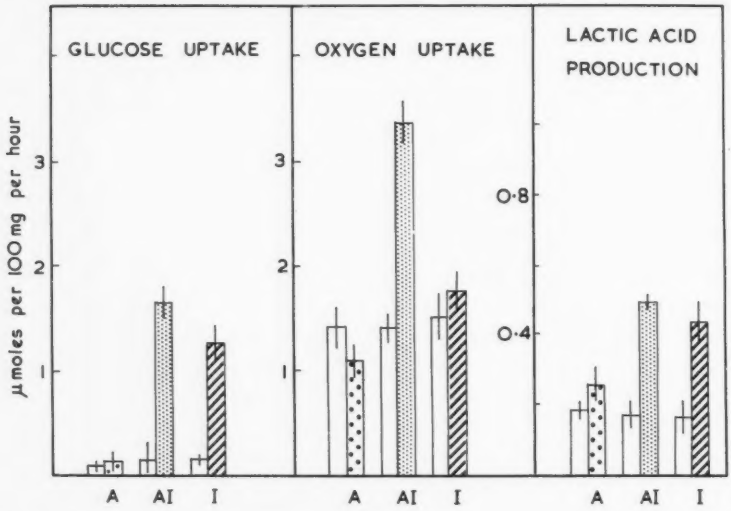

FIG. 5. The effects of adrenaline and of insulin on the glucose uptake, oxygen uptake and lactic acid production by rat epididymal adipose tissue.

The incubation medium was glycylglycine-buffered Ringer's solution containing glucose, 90 mg. per 100 ml. Adrenaline concentration was 10 μg per ml. Insulin concentration was 0.1 units per ml. Incubation period was 3 hours. The glucose and stimulating agent were added to the tissue at zero time. Glucose uptake, oxygen uptake and lactic acid production are expressed as μmoles per 100 mg. tissue per hour; they are calculated from the value obtained at the end of three hours' incubation. The bars represent the mean of 5 experiments; the lines represent the standard errors of the means. A = adrenaline, I = insulin, AI = adrenaline + insulin. The unlabeled bars are the control values.

fumarate, aspartate, glutamate, glutamine) only pyruvate caused any increase in oxygen consumption, and the addition of adrenaline to tissue incubated with pyruvate caused a further increase in oxygen uptake. In a representative experiment with paired tissues pyruvate, 0.4 mM, increased the oxygen uptake of adipose tissue from 32.6 μl. per 100 mg. tissue per hour to 50.6 μl. The addition of adrenaline to both tissues increased the oxygen uptakes to 34 μl. per 100 mg. tissue per hour in the tissue incubated without pyruvate and to 86 μl. in the tissue incubated with pyruvate. This effect of pyruvate on the oxygen consumption of adipose tissue is being investigated further.

DISCUSSION

The present results confirm the observations of Lynn and co-workers (10) and of Orth and co-workers (11) that adrenaline increases oxygen uptake by adipose tissue and the observation of Cahill and co-workers (12) that adrenaline increases glucose uptake by adipose tissue incubated with albumin.

The inhibition of oxygen uptake by adrenaline was also observed by Lynn *et al.* (10) and the present results suggest that this may be due to the intracellular accumulation of FFA. That adrenaline causes the intracellular accumulation of FFA and that this accumulation is smaller when an FFA acceptor such as albumin is present in the medium was shown by Engel and co-workers (13) and has been confirmed by us. There is a good correlation between the concentration of FFA in the tissue and the inhibition of oxygen uptake by adrenaline. Long chain fatty acids are known to be potent uncoupling agents (14) and to inhibit electron transport when present in high concentrations (15) (16). The possibility exists that the increase in oxygen uptake seen with adrenaline under certain conditions may be partly due to an uncoupling of oxidative phosphorylation by FFA.

The substrate oxidized under the influence of adrenaline is, at least partly, glucose. Cahill, LeBoeuf and Flinn (12) have shown that adrenaline increases glucose oxidation in the tricarboxylic acid cycle in adipose tissue and apparently decreases the metabolism of glucose via the pentose phosphate oxidative pathway. In the presence of insulin more glucose is metabolized but the pathway is very similar to that seen with adrenaline alone (12) resulting in an even greater oxidation of glucose. The present results are in agreement with these observations. However, the oxidation of fatty acids cannot be excluded; it is known that fatty acids are oxidized by adipose tissue (17) (18) and that adrenaline increases fatty acid oxidation in liver (19) (20).

In previous publications it has been shown that growth hormone preparations (9) and glucagon (21) also increase the oxygen consumption of adipose tissue and with both hormones glucose is required for this effect. Both glucagon (22) (21) and growth hormone (23) release FFA from adipose tissue. Clearly there is a relationship between increase in FFA release and increase in oxygen consumption. Cahill, LeBoeuf and Flinn (12) have suggested that the effects of adrenaline on glucose metabolism by adipose tissue may be mediated entirely by FFA since increasing the FFA content of the tissues by adding FFA externally also produced the same pattern of glucose metabolism as does adrenaline.

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SPECIFICITY OF THYROXINE ACTION ON OXYGEN UPTAKE OF KIDNEY SLICES DURING PROLONGED INCUBATION[1]

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ABSTRACT

The effect of a number of thyroxine analogs has been evaluated using kidney tissue respiration during prolonged incubation. Thyroxine was found to be the most active compound studied. All substances with marked thyroxine-like activity had a skeletal structure of a diphenyl ether with at least three substitutions on the rings. Iodo, methyl, bromo, chloro and nitro substitutions activated with decreasing effectiveness in that order. Variation in the number of carbon atoms, alteration of the optical configuration of the alanine side chain or its replacement by a short-chain fatty acid caused only slight reduction of the activity of an analog, as did the replacement of the ether oxygen with a sulfur. The results can be correlated with those from *in vivo* experiments and suggest that the thyroxine effect on tissue respiration in this type of system may yield information of value in explaining the hormone's mechanism of action in whole animals.

IN 1956 Barker (1) developed an *in vitro* system in which the cellular action of thyroxine (T4) was demonstrated by maintenance, or even enhancement, of oxygen consumption by kidney slices during prolonged incubation. Such a system, being intermediate in complexity between the whole animal and broken cell preparations, offers another tool for studying the relationship between thyroid hormones and cellular metabolism. With some *in vitro* systems, "thyroxine action" as measured by enhancement of succinoxidase (2), ascorbic acid oxidase (3), or uncoupling of oxidative phosphorylation (4, 5), can be elicited by many thyroxine analogs and other unrelated compounds without regard to their metabolic effect in animals. Therefore, we have compared the effects of a number of thyroxine analogs on the oxygen uptake of kidney slices in order to elucidate the limits of structural specificity for thyroxine-like action in this system.

METHODS

The experimental conditions were essentially the same as those described by Lindsay and Barker (6). Sprague-Dawley rats weighing 300–400 grams, at least six weeks after

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thyroidectomy, were sacrificed by exsanguination. Both kidneys were removed, chilled and slices of cortex prepared according to the Deutsch technique (7 p. 136). About 60 mg. were weighed on a torsion balance for each Warburg vessel containing Krebs' Ringer solution with 200 mg.% glucose and 0.018M L-proline. Thyroxine or an analog was tested at 1.3×10^{-5}M in 3 ml. of incubation medium at a final pH of 7.45; blank controls contained the same quantity of 0.005 N NaOH. All solutions were added at the same time just before incubation to insure equal contact time between the tissue and the testing analogs among all vessels.

After oxygenation and a 15 minute period of equilibration at 37° C, the tissue oxygen consumption was measured for one hour. The vessels were then flushed with oxygen, capped with Parafilm® and stored at 5° C until the following day when the tissue oxygen consumption was again measured. This same procedure of incubation at 37° C and cold

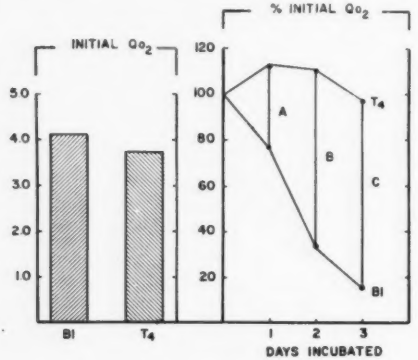


FIG. 1. Oxygen consumption of kidney slices incubated in Ringer-proline-glucose solution with and without thyroxine. On the left, the initial Q_{O_2} values are averaged for 12 experiments, showing control (B1) as the plain bar and thyroxine (T4) as the cross-hatched. On the right is shown the metabolic activity, expressed as % of initial Q_{O_2}, of tissue incubated in B1 and T4 for 1, 2 and 3 days, as described in the text. The sum of lines a, b and c (T4 "maintenance value") was used as the 100% T4 reference activity in evaluating various analogs.

storage at 5° C was repeated once every 24 hours for 3 days. Each experiment was run in duplicate and was controlled by its own blank and thyroxine vessels. The tissue respiration was calculated as Q_{O_2} in μl. oxygen/mg. wet tissue/hour.

RESULTS

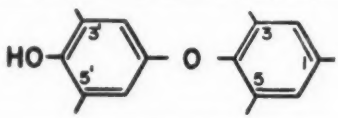
Figure 1 gives the average values from 12 experiments with T4. As it indicates, the T4 enhancement of oxygen uptake by kidney tissue became apparent after 24 hours, the initial Q_{O_2} being quite similar, 4.12 and 3.74 μl./mg./hr. in the blank and thyroxine vessels, respectively. The respiration in the thyroxine vessel rose to 112% of its initial Q_{O_2} after the first day and was still 97% at the end of three days while the oxygen consumption of the control fell sharply to 15% at the end of 3 days' incubation. In each experiment, these differences in oxygen uptake (pictured here by lines a, b and c) were considered as the net T4 "maintenance effect" on tissue respiration. This summated value was used in each case as the 100% basis for calculation of thyroxine-like activity of analogs.

In general, compounds lacking the diphenyl ether skeletal structure gave essentially no maintenance of tissue respiration in this system. N-Acetyl-diiodotyrosyl-diiodotyrosine displayed 15% of thyroxine activity, N-(4′-hydroxy-3′,5′-diiodobenzoyl)-3,5-diiodotyrosine 13% and tetra-

iodophenolphthalein 0%. Diiodotyrosine and monoiodotyrosine showed 7% and 0%, respectively.

Among the different iodinated thyronines, thyroxine was uniformly the most active compound. The activity of the analogs diminished in a regular fashion with decreasing number of iodine substitutions on the diphenyl ether. As shown in Table 1, 3,5,3′-triiodothyronine (3,5,3′-T3) and 3,3′,5′-triiodothyronine (3,3′,5′-T3) both exhibited 83% of T4 activity. These values are at variance with observations in intact mammals in which 3,5,3′-T3 displayed 4–8 times the T4 activity and 3,3′,5′-T3 had no significant activity (8, Pittman and Barker, unpublished data). The lower

TABLE 1. COMPARISON OF MAINTENANCE OF KIDNEY SLICE METABOLISM BY VARIOUS IODINATED ANALOGS OF THYROXINE



Substitutions at 3,5,3′,5′	Substitution at 1				
	Alanine	Butyric	Propionic	Acetic	Formic
3,5,3′,5′-Tetraiodo	100.0[1]	82.1	95.7	82.9	79.4
3,5,3′-Triiodo	82.9		76.8	90.8	
3,3′,5′-Triiodo	82.9				
3,5-Diiodo	42.2		50.9	50.7	
3,3′-Diiodo	58.0		48.0	51.8	
3-Monoiodo	47.0				
3,5,3′,5′-Tetrahydro	4.5				

[1] All figures are expressed as % of thyroxine maintenance value.

value of 3,5,3′-T3 agrees with reports from other *in vitro* systems under quite dissimilar conditions (2, 4, 9, 10). In our incubation system, the thyronines with only two iodine substitutions, the 3,3′- and 3,5-diiodothyronine showed 58% and 42% thyroxine activity. Thyronine, having hydrogen in all four 3,5,3′ and 5′ positions, showed an insignificant 5% activity. A similar diminishing order of activity according to a decreasing number of iodine substitutions also held true among the fatty acid analogs.

Replacement of the alanine side chain of iodothyronines with fatty acids from formic to butyric slightly reduced the thyroxine-like activity. In general, these analogs exhibited higher activity in our *in vitro* system than in whole-animal experiments (10, Pittman and Barker, unpublished data), although none was more active than T4 itself, as was often observed in assays by tadpole metamorphosis (11, 12). For example, by tadpole assay, 3,5,3′,5′-tetraiodothyropropionic acid (Pr_4) was reported by Money and colleagues to possess 8,000 times T4 activity while in our laboratory its calorigenic activity in thyroidectomized rats was found to be about 5% that of T4. In our kidney incubation system, as seen in Table 1, Pr_4 and

3,5,3′,5′-tetraiodothyroacetic acid showed 96% and 83% of T4 activity, respectively. Either lengthening or shortening the side chain by one carbon atom exerted no significant effect on the activity; 3,5,3′,5′-tetraiodothyrobutyric acid displayed 82% activity and 3,5,3′,5′-tetraiodothyroformic acid 79%. Thus, while the optimal length of side chain still appeared to be 2 or 3 carbon atoms in these experiments with proline, the relationship of its length to thyroxine activity of the analog was not nearly as dramatic as was seen with alanine as substrate, or in the results using whole animals (13).

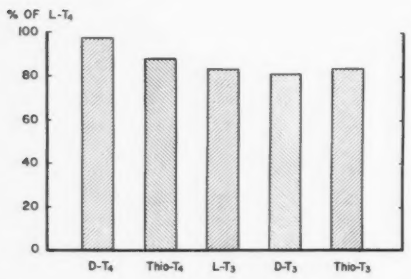


FIG. 2. Effect of optical configuration and replacement of ether oxygen with sulfur on thyroxine and triiodothyronine maintenance of kidney slice metabolism. The activity of each of the following compounds is expressed as % of the L-thyroxine (L-T4) maintenance value: D-thyroxine (D-T4), 3,5,3′-triiodo-L-thyronine (L-T3), 3,5,3′-triiodo-D-thyronine (D-T3), 3,5-diiodo-4-(4′-hydroxy-3′,5′-diiodo-phenylmercapto)-DL-phenylalanine (Thio-T4) and 3,5-diiodo-4-(4′-hydroxy-3′-iodophenylmercapto)-DL-phenylalanine (Thio-T3).

D-Thyroxine (D-T4) has long been known to have only a few percent of the L-T4 calorigenic action in the whole animal (14), although both optical isomers were found equally effective in uncoupling oxidative phosphorylation or promoting swelling of mitochondria (10, 15). Our system also showed little preference for any one optical isomer (Fig. 2): D-T4 displayed 98% as much activity as L-T4, and D- and L-T3 82% and 83%, respectively.

Replacement of the ether oxygen of thyroxine or T3 by a sulfur gives rise to 3,5-diiodo-4-(4′-hydroxy-3′,5′-diiodophenylthio)-phenylalanine (Thio-T4) or 3,5-diiodo-4-(4′-hydroxy-3′-iodophenylthio)-phenylalanine (Thio-T3), respectively. In our system (Fig. 3), Thio-T4 displayed 88% thyroxine activity and Thio-T3, 84%. Both values approximate their respective oxygen analogs and agree with the published results obtained from uncoupling of phosphorylation (10) or enhancement of succinoxidase (2). However, when Thio-T4 was assayed using myxedematous patients, Lerman and colleagues reported no more than 10–13% thyroxine activity (16).

The results obtained from T4 analogs containing chlorine, bromine, methyl and nitro groups in place of iodine are summarized in Figure 3. Next to iodine, the methyl group was by far the most effective activator of thyronine molecule: 3,5,3′,5′-tetramethyl-DL-thyronine (Me_4T) showed 92% as much activity as T4. By BMR assay, Me_4T had no demonstrable calorigenic effect in thyroidectomized rats (17). Further comment will be

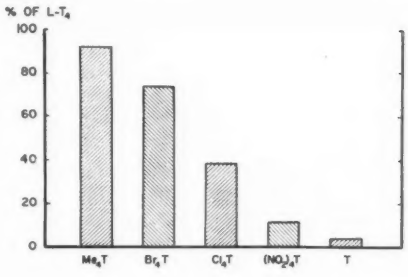


FIG. 3. Effect of replacement of iodine on thyroxine maintenance of kidney slice metabolism. The activity of each of the following compounds is expressed as % of the L-thyroxine (L-T4) maintenance value: 3,5,3′,5′ - tetramethyl - DL - thyronine (Me_4T), 3,5,3′,5′-tetrabromo-DL-thyronine (Br_4T), 3,5,3′,5′-tetrachloro-DL-thyronine (Cl_4T), 3,5,3′,5′-tetranitro-DL-thyronine ($(NO_2)_4T$) and L-thyronine (T).

made in conjunction with Figure 4. Figure 3 also shows that replacement of the four iodines in T4 by other halogens diminished the metabolic effects on incubated kidney tissue. 3,5,3′,5′-Tetrabromo-DL-thyronine (Br_4T) gave 75% of T4 activity and 3,5,3′,5′-tetrachloro-DL-thyronine (Cl_4T) 39%. 3,5,3′,5′-Tetranitro-DL-thyronine, $(NO_2)_4T$, displayed no significant activity (11%). These results parallel published assays in whole animals (8, 14, 18), although the first two are considerably higher *in vitro*.

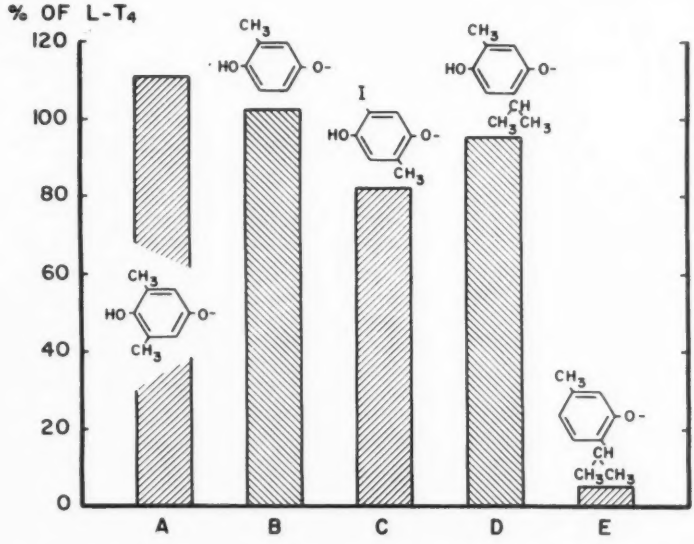


FIG. 4. Effect of alkyl substitutions at positions 3′,5′ and 6′ on 3,5-diiodothyronine maintenance of kidney slice metabolism. Metabolic activity on the ordinate is expressed as % of L-T4 maintenance. At each bar is shown the specific 4-(3′,5′- or 6′-alkyl-phenoxy) group to be joined with 3,5-diiodo-phenylalanine to yield, respectively: (A) 3′,5′-dimethyl-3,5-diiodothyronine; (B) 3′-methyl-3,5-diiodothyronine; (C) 3′-iodo-6′-methyl-3,5-diiodothyronine; (D) 3′-methyl-6′-isopropyl-3,5-diiodothyronine; (E) 3′-methyl-4′-deoxy-6′-isopropyl-3,5-diiodothyronine. Note that the last compound is not a true thyronine, since it is lacking the 4′-hydroxyl.

In addition to tetramethyl-thyronine, just discussed, other methylated thyronines have been evaluated, all containing iodines in the 3,5-positions. From Figure 4, it is apparent that both 3′,5′-dimethyl- and 3′-monomethyl-3,5-diiodothyronine were fully as active as Me_4T and T4 itself. As already noted, Me_4T is inactive *in vivo*, in contrast to the positive results obtained with the methyl-diiodothyronines both in the present study (Fig. 4) and in thyroidectomized animals (17). Metabolic activity is thus not specifically dependent upon the presence of iodine in the 3′,5′-positions. Obviously, there is some unique *in vivo* importance of iodine over methyl 3,5-substitution which seems lacking in these kidney slice experiments.

The results presented in bars C and D of Figure 4 reveal that 6′-substitution of a methyl or isopropyl group did not significantly alter the metabolic activity of 3′-iodo- or 3′-methyl-3,5-diiodothyronine, respectively. The basal metabolic rate responses of thyroidectomized rats to these compounds have been found blocked to a considerable extent by the 6′-substitution (Shida, Shimada and Barker, unpublished). There is thus another subtle difference between specific thyroxine structural requirements *in vivo* and *in vitro*.

The 4′-hydroxyl group has been considered necessary to enable T4 to act as a chelating agent (19, 20). Blockade of the free hydroxyl by formation of the methyl ether has been variously reported as increasing or decreasing T4 activity, probably because of differing solubilities. Column E of Figure 4 shows that removal of the 4′-oxygen from the fully active 3′-methyl-6′-isopropyl-3,5-diiodothyronine (D) completely abolished its *in vitro* metabolism maintaining characteristics. We have obtained a similar lack of activity in 4′-deoxy-3′,6′-dimethyl-3,5-diiodothyronine, although it is not shown in the figure, since the corresponding 4′-hydroxy compound has not been tested. Jorgensen (21) has reported detectable levels of T4-like antigoitrogenic acitivity in these and related 4′-deoxy compounds. Further evaluation, both *in vitro* and *in vivo* is required, and the most important single compound, 4′-deoxy-T4, has not yet been synthesized.

DISCUSSION

These results evaluating T4 analogs on an "intact cell" preparation, rat kidney cortex slices, lend further support to the concept that thyroxine activity resides primarily in the structural moiety of a p′-hydroxy-diphenyl ether with two or more halogen or small alkyl substitutions on the rings. Aside from a few outstanding discrepancies which are discussed below, our results generally paralleled the observations from whole animal assays, although the relative effects on tissue respiration tend to be greater.

It has been suggested that the greater activity shown by T3 over T4 in intact animals corresponds to its weaker bond with the thyroxine-binding proteins of plasma and hence its greater ease of entry into the cells (22, 23). This view is supported by Newcomer (24) and by Tata and Shellabarger

(25) who found that in domestic fowl whose plasma lacked specific thyroxine-binding proteins, T4 and T3 showed similar biological half-lives as well as equal calorigenic activity. A comparable situation probably existed in our incubation system in which a medium originally free of protein was employed. Although some "leaking" of tissue protein can be shown to occur during incubation of kidney cortex slices, such loss is not great and it is questionable that the effective tissue concentration of T4 and T3 could be altered. As a result, there was no enhancement of T3 activity over that of T4.

On the other hand, 3,3′,5′-T3, known to have no significant calorigenic action *in vivo*, here displayed activity equal to T3. Taken in conjunction with the lower, but appreciable activity shown by other partially-iodinated thyronines (Table 1) as well as those carrying bromine or chlorine (Fig. 3), these findings point to a considerable variety of responsiveness of the *in vitro* system to substitutions at 3 and 5 on the "inner" ring of the dephenyl. The situation was commented on earlier with reference to tetramethyl thyronine, inactive *in vivo*, but fully as effective *in vitro* as T4.

Dunne and Tapley (26) studied the oxygen consumption of a number of excised tissues from rats previously treated with D-T4 and reported that while the Q_{O_2} of liver and kidney slices showed a definite rise over their control values, no change of Q_{O_2} was observed in heart or diaphragm. This lack of enhancement of the respiration of striated muscles, which in rats contribute approximately 50% of the total body weight, was used by these workers to explain the low calorigenic effect of D-T4 in whole animals and the equal activity of both optical isomers of T4 under *in vitro* conditions as observed in our systems.

The foregoing discussion reminds one of the fact that in a living animal, analogs of closely related structure may be handled very differently by a multitude of normal physiological processes such as absorption, transport, degradation and excretion, whose relative influence on the effective tissue concentration of these analogs must change under *in vitro* conditions. This is clearly reflected by the discrepancies in the relative activity of thyroxine analogs when assayed by systems of varied complexity and such discrepancies can be reconciled only when we have more detailed information about the metabolic pathways of these compounds. Our kidney slice system being intermediate between the whole animal and broken cell preparations has given results intermediate between the two experimental conditions. They suggest that it may eventually be possible to obtain genuinely revealing insight into the specific mechanism of T4 action.

Acknowledgments

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maining iodothyronines and all of the fatty acid analogs studied were furnished through the generosity of Drs. R. I. Meltzer and R. L. Kroc of the Warner-Lambert Research Institute. Dr. R. Pitt-Rivers made available the tetrabromo- and tetrachlorothyronines.

3,5,3′,5′-Tetramethyl-DL-thyronine and 3,5-diiodo-3′,5′-dimethyl-DL-thyronine were gifts from Dr. J. Wolff of the National Institutes of Health who had obtained these analogs respectively from Dr. H. J. Bielig of Heidelberg, Germany and the Glaxo Laboratories, England. 3,5-Diiodo-3′-methyl-DL-thyronine was obtained from Dr. N. S. Kharasch of the University of Southern California. The rest of the alkylated analogs were kindly furnished by Dr. Jorgensen of University of California, San Francisco.

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EFFECTS OF GROWTH HORMONE AND CORTICOSTEROIDS ON S^{35} FIXATION IN CARTILAGE

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ABSTRACT

Previous studies on the effect of growth hormone on S^{35} fixation in cartilage have been extended to learn more about this reaction and to apply it as a possible assay for growth hormone. It had been noted that increased dosages of growth hormone were accompanied by increased levels of S^{35} incorporated into costal cartilage of the hypophysectomized rat.

The duration of treatment necessary to produce a significant stimulation in S^{35} uptake was 48 hours. Results after four days of treatment were nearly maximal. The effective biological life of circulating growth hormone as measured by the incorporation of S^{35} in cartilage is from 24 to 48 hours. The disappearance curve reflecting the metabolic activity of S^{35} containing compounds in cartilage closely approximates the previously reported half-life of radiochondroitin sulfate, that is, seventeen days. The use of human serum as a vehicle for growth hormone produces no modification of its action, while the use of ten percent gelatin as a vehicle results in a slight decrease in S^{35} binding. The subcutaneous route of administration of the hormone is as effective as the intraperitoneal route.

Hydrocortisone inhibits S^{35} fixation to about one half of the level noted in control animals. Hydrocortisone also inhibits the stimulatory action of growth hormone, and conversely growth hormone antagonizes the action of hydrocortisone. Cortisone and 9-fluohydrocortisone behave similarly to hydrocortisone in reducing S^{35} incorporation into cartilage and in reducing the increase in body weight. Hydrocortisone hemi-succinate and tetrahydrocortisone had no notable effect on these processes.

PREVIOUS studies have demonstrated that growth hormone enhances the uptake of S^{35} by the costal and xyphoid cartilages and by the tibial cap of the hypophysectomized rat (1, 2, 3). This process is presumably intimately related to the long-known chondrotrophic properties of the growth hormone as recognized morphologically (4). The present report extends our description of the effects of growth hormone in respect to the

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time at which the increment in S^{35} binding begins, the time of maximum influence of a constant dose of growth hormone and the duration of influence when the growth hormone administration is stopped.

Since cortisone and hydrocortisone have been shown to interfere with the response of several tissues including cartilage to the growth hormone this effect is studied in terms of S^{35} incorporation. A generalized decrease of S^{35} fixation in tissues has already been noted in radioautographs of rats that have received cortisone systemically (5) and a decreased binding of S^{35} has been observed in rat cartilage in vitro when cortisone was added to the medium (6).

Chondroitin sulfate is the chief repository of accumulated S^{35} in cartilage, although it may not be the only one (7, 8, 9). Degradation curves of the material deposited under the influence of growth hormone in hypophysectomized rats are given as is their comparison to those of radioactive chondroitin sulfate.

Since the sensitivity of any response to the growth hormone is of interest to those concerned with assay possibilities and since a dose response curve is necessary both for assay and for comparison with other growth responses a preliminary dose response curve for S^{35} uptake is included. In view of the spread of individual responses a final decision on the usefulness of this method cannot be undertaken now. The suitability of human serum as a vehicle for growth hormone was studied as was the effect of a gelatin vehicle. The influence of route of administration of hormone, subcutaneous or intraperitoneal, was also investigated.

EXPERIMENTAL

Modifications of the original procedure (2) were made for various phases of study. Young female rats were used as experimental animals. Hypophysectomy was done at age 22 days, shortly after weaning. Litter mates served as controls. Animals were usually used experimentally on the fifth day post-operatively. In studies on the duration of treatment with growth hormone necessary to produce stimulation of S^{35} binding in cartilage the animals were used experimentally on the fourteenth day post-operatively. In this experiment and in the dose-response study the animals received radioactive sulfur in 10 μc doses in saline while all other experiments were done with 5 μc doses. Correspondingly in animals receiving 5 μc of S^{35} the carrier sulfate was decreased by one half using 5 ml. of 0.05N Na_2SO_4 and 3 ml. of 0.5N $BaCl_2$. The growth hormone used was Armour, Lot 411032R. The radiosulfur was obtained from Oak Ridge National Laboratories, Oak Ridge, Tennessee, as carrier-free sulfate. All experiments were repeated at least three times.

Hormone solutions were made in convenient concentration so that the required dosage could be administered in volumes of 0.5 or 1.0 ml. The steroids, hydrocortisone acetate, cortisone acetate and tetrahydrocortisone, were administered in saline suspension. Hydrocortisone-hemissucinate was dissolved in saline. Control animals received saline equal in volume to that of the corresponding hormone solution being tested. Growth hormone was also dissolved in human sera, 100 μg in 0.25 ml. and in 10% gelatin solution. Control animals received corresponding volumes of the vehicle in these studies.

In experiments involving the duration of treatment necessary for growth hormone

timulation and in the dose-response study, animals were sacrificed 48 hours after the st dose of sulfur and growth hormone. Suitable tissues were removed, cleaned and dried n air before ashing. In the other experiments animals were sacrificed 24 hours after the st dose of hormone and sulfur. Tissues were processed similarly and radioactivity deter-ined on the basis of wet weight of the tissues. Costal cartilage and tibial caps were udied in all groups, although only the costal cartilage data are presented in the graphs. sually three samples of costal cartilage were obtained from each animal and the aver-ge of these figures was used. However, in the growth hormone-dose response experi-ents groups of three to five rats are combined at each dose level with the average figure r the group being used. Cardiac muscle was examined repeatedly as a check against ntamination with extraneous radiosulfate. Correction was made for radio-decay of S^{35}.

In the study of the duration of effect of circulating growth hormone the animals eceived one dose of S^{35}, 15 μc at varying periods, 24, 48, 72 and 96 hours after the last ose of growth hormone. In the study of the rate of disappearance of radiosulfated com-ounds in cartilage, animals were observed for a period of twenty-one days.

Our determinations of S^{35} concentration were always made at least 24 hours after the st injection of the isotope. Thus no information was obtained on the influence of growth hormone on more labile substances. Only the behavior of the more stable compounds retaining S^{35} for at least 24 hours can be compared with that of chondroitin sulfate.

RESULTS

The Duration of Growth Hormone Treatment Necessary to Produce Stimulation of S^{35} Fixation in Cartilage

There was no noticeable effect on S^{35} fixation in cartilage following a single injection of 100 μg of growth hormone. Growth hormone given at the level of 100 μg per day for two days produced a notable two- or three-fold stimulation of S^{35} fixation in the costal cartilage of the hypophysectomized rat (Fig. 1). In three days, the effect was more marked. Treatment for four days produced an effect that was somewhat higher than that observed after treatment for three days and practically as high as that observed in animals treated for eight days. Animals receiving S^{35} and growth hormone for eight days had S^{35} fixation slightly higher than the animals treated for four days. The plateau of growth hormone effect at this dose level is evidently reached in about four days. Increases in body weight were noted also during the period of growth hormone treatment. Growth hormone-treated animals gained an approximate average of 2 gm. per day, while the untreated controls gained an average of less than 1 gm. per day during the experimental period.

Duration of Effect of Circulating Growth Hormone

Growth hormone remained effective in the circulation of the hypophysectomized rat approximately 24 hours after administration as determined by its effect on stimulating S^{35} fixation in costal cartilage (Fig. 2). This effect is practically absent 48 hours after the last injection of growth hormone indicating a relatively short biological life for the circulating growth hormone. The combined effect of metabolism and excretion of the

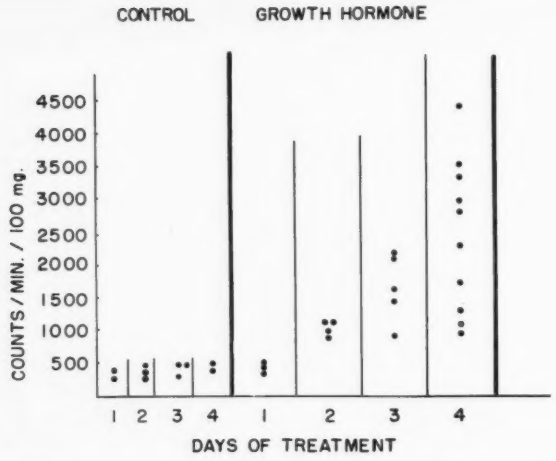

FIG. 1. Duration of growth hormone treatment necessary to stimulate S^{35} fixation. (Denko and Bergenstal.)

hormone cannot further be elucidated by these data. The increase in weight of the animals noted during the period of growth hormone activity did not persist after the growth hormone treatment was terminated.

The Disappearance Rate of S^{35} Bearing Compounds in Cartilage

The disappearance rate of S^{35} containing compounds in the cartilage is not influenced materially by prior treatment of hypophysectomized rats

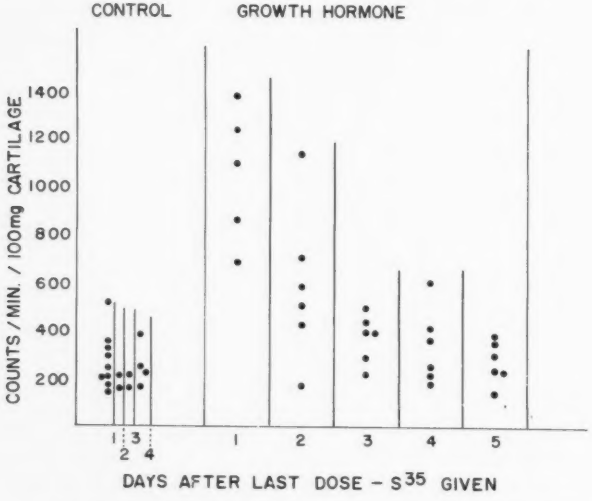

FIG. 2. Duration of effect of circulating growth hormone on S^{35} fixation. (Denko and Bergenstal.)

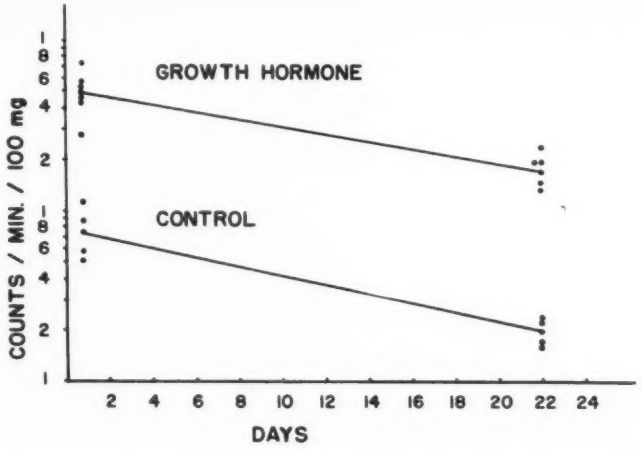

FIG. 3. Disappearance curve of S^{35} compounds in cartilage. (Denko and Bergenstal.)

with growth hormone (Fig. 3). The slope of this curve in growth hormone-treated animals is similar to the slope of the curve observed in the untreated or control animals. Disappearance curves of S^{35}-compounds in cartilage of animals receiving treatment for an eight day period were similar to those curves reflecting the disappearance of S^{35} compounds in cartilage of animals receiving the four day treatment. The spread in individual animal values is such that only these general observations can be made with these data.

The Effect of Increasing Growth Hormone Dose on S^{35} Fixation

In animals receiving increasing doses of growth hormone there is an increasingly greater fixation of S^{35} in the cartilage (Fig. 4). The response to increased dosage approximates a straight line in the dosage range of 0 to 1000 μg. There is considerable variation in animal response in the lowest doses, below 5 μg.

The Effect of Route of Administration and Vehicle

Subcutaneous injection of growth hormone in saline produced results similar to those observed in animals that received intraperitoneal injections of growth hormone and S^{35}. The use of 10% gelatin as a vehicle for growth hormone and S^{35} administration was of no practical value. Animals that were given hormone dissolved in gelatin had a somewhat lower fixation of S^{35} in the cartilage than did those given hormone in saline. The stimulation of weight gain was somewhat greater in animals receiving growth hormone in saline than that in animals receiving growth hormone dissolved in gelatin.

When growth hormone was dissolved in human plasma and injected intraperitoneally in doses of 100 μg in 0.25 ml. of plasma there was no

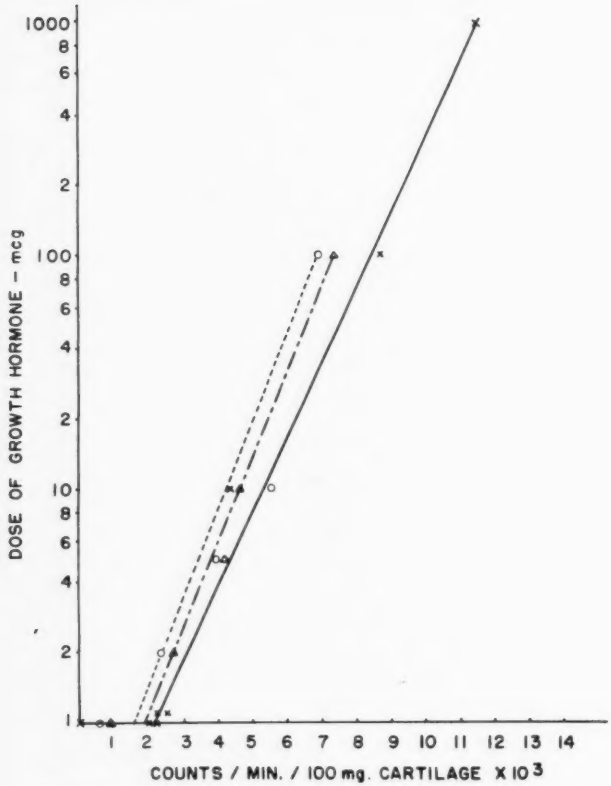

FIG. 4. The effect of increasing growth hormone on S^{35} fixation in cartilage. (Denko and Bergenstal.)

TABLE 1. THE EFFECT OF HYDROCORTISONE ON THE S^{35} FIXATION IN THE COSTAL CARTILAGE OF UNTREATED AND GROWTH HORMONE-TREATED HYPOPHYSECTOMIZED RATS

Animals	Group	Dose μg	c.p.m.*	Significance of comparison
9	C—Control		735 ± 225	P < 0.01
13	HC—Hydrocortisone	25–100	390 ± 127	
5	GH—Growth Hormone	50	3,387 ± 1,035	P < 0.02
5	GH + HC	GH—50 + HC—100	1,455 ± 799	
13	HC—Hydrocortisone	25–100	390 ± 127	P < 0.01
5	HC + GH	HC—100 + GH—50	1,455 ± 799	

* c.p.m.—counts per minute per 100 mg. cartilage.

notable difference in the response of costal cartilage in fixing S^{35} when compared to animals receiving 100 μg of growth hormone dissolved in saline.

The Effect of Steroids in S^{35} Fixation in Cartilage and on the Growth Hormone Stimulation of S^{35} Binding in Cartilage

In animals receiving hydrocortisone there was a marked reduction in the fixation of S^{35} in the costal cartilage (Table 1). The mean values of S^{35} uptake in animals receiving 25 to 100 μg of hydrocortisone acetate ranged from $\frac{1}{3}$ to $\frac{1}{2}$ lower than the mean values in control animals. The inhibitory effect of hydrocortisone is pronounced in its effects on the stimulatory action of growth hormone on S^{35} fixation. In fact in some animals the values were lowered to values obtained in the untreated control animals. The stimulatory action of growth hormone counteracts the inhibitory effect of hydrocortisone. Animals receiving cortisone in 2.5 mg. doses also had a marked reduction in S^{35} incorporated into the cartilage. 9-Fluoro-hydrocortisone in 100 μg also caused an inhibition of the S^{35} binding. Tetrahydrocortisone had no effect on the S^{35} uptake as did the hemisuccinate of hydrocortisone, both given in doses ranging to 1000 μg daily.

The catabolic effect of 100 μg of hydrocortisone on weight gain is reflected by the weight loss of 3 gm./rat noted during the experimental period of 4 days. Control animals gained two grams while the growth hormone- and hydrocortisone-treated animals gained approximately the same increment as the rats receiving only growth hormone. Thus, while the weight gains of growth hormone-treated animals are only slightly or inconclusively retarded by hydrocortisone, the inhibitory effect of hydrocortisone on the sulfur binding of their costal cartilage is much more dramatic than the effect on weight gain.

DISCUSSION

Growth hormone appears to be relatively short-lived. Investigators using the width of tibial epiphyseal cartilage as a measure of growth hormone have studied the concentration in the plasma of hypophysectomized rats. They estimate only one-third of the injected dose could be found immediately after injection. The biological half-life of growth hormone was reported to be only 40 minutes following the intravenous injection of a single dose of growth hormone (10).

By isolating S^{35}-containing chondroitin sulfate from the cartilage of animals that had received injections of inorganic S^{35}-sulfate, Boström noted that over a period of 17 days the amount of radioactive chondroitin sulfate had dropped to one-half of the level observed originally (9). This period of 17 days, termed the half-life of chondroitin sulfate, reflects a complex process summarizing synthesis and re-utilization of sulfur as well as degradation of the observed radioactive compound in a stable system.

The theoretical disappearance rate curve for radiochondroitin sulfate, i.e., loss of one half of the radioactivity in 17 days, is observed to approximate our experimental disappearance curve. This indicates that the compounds formed, when S^{35} is incorporated into the cartilage, are largely chondroitin sulfate or behave like chondroitin sulfate. More than one compound containing radiosulfate may be present.

Growth hormone apparently does not alter the fundamental nature of the S^{35}-containing compounds formed in cartilage since both the untreated and treated animals had similar disappearance rates. Growth hormone appears to increase the rate of formation of the radiosulfated compounds much more than it increases the rate of weight increase of the animal.

The turnover or disappearance curve represents a complex process involving most likely at least the processes of synthesis and degradation. Net synthesis of radiosulfate compounds occurs when the synthetic or anabolic operation is predominant while degradation occurs when the degradative or catabolic operation is predominant. The primary alteration in the turnover process may be due to an increase in the net synthesis of radiosulfate compounds. A more exact determination of the site of action in relation to the synthetic process is not possible at this time. A number of mechanisms may be implicated, e.g. an increase in utilization of the molecular components of chondroitin sulfate or an increase in the mass of the enzyme system responsible for synthesis.

The effect of hydrocortisone on S^{35} incorporation in cartilage may be due to decreased synthesis of radiosulfate-compounds or an increased degradation of this material or perhaps to both processes. In hydrocortisone treated animals a decreased synthesis is more likely since at no times were values found that approximated the values in control animals. The inhibitory action of hydrocortisone on S^{35} binding in cartilage remains clear as does the marked inhibitory effect on the stimulation of S^{35} uptake provided by growth hormone. Conversely the stimulatory effect of growth hormone on this process counteracts the activity of hydrocortisone in decreasing S^{35} fixation.

The steroids of the cortisone family, namely cortisone, hydrocortisone, and 9-fluorohydrocortisone, interfere both with S^{35} binding process and with increase in body weight. Tetrahydrocortisone, the metabolic product of cortisone degradation in the body, was found to be without effect. The hemisuccinate of hydrocortisone was also without effect. Perhaps this compound is excreted too rapidly to produce a detectable effect or it may not be bound by the serum proteins and circulated to effective sites of action. It may not be hydrolyzed sufficiently to make the hydrocortisone effective.

Since an increase in S^{35} fixation occurs with an increase in dosage of growth hormone it may be possible to adapt this technique for assaying growth hormone especially in the higher concentrations. Unfortunately the greatest variation in results occurs in the lowest dose levels studied,

below 5 μg, thus decreasing the sensitivity of this technique in detecting low level material. At higher levels, 10 μg or more, there is a marked stimulation of S^{35} incorporation.

More work must be done to rule out the influence of interfering compounds in materials that may be subject to assay. A summary of published investigations of the effects of other hormones on S^{35} incorporation in costal cartilage reveals that thyroxine (11) and testosterone propionate (12) stimulate radiosulfate incorporation while cortisone (6) and hydrocortisone (3, 12) inhibit this activity of the cartilage. No stimulatory effects of thyroid stimulating hormone were noted on studies utilizing thyrotrophin (2). Large doses of prolactin, one unit or more, have been shown to increase radiosulfur fixation in cartilage (13), while small doses of prolactin do not (13, 14), nor does ICSH, interstitial cell stimulating hormone, (14) or FSH, follicle stimulating hormone, (14). A direct comparison of the S^{35} incorporation method with that of the tibia assay indicates that the radiosulfate uptake assay is more precise and specific than the tibia assay (15).

A wide variety of biochemical systems have been reported influenced by growth hormone. Only the over-all resultant effect can be evaluated since the specific action or actions influencing the biochemical indicator system has not been identified. However, the various mechanisms or enzyme systems involved in the radiosulfate incorporation reactions are sensitive and respond measurably within a matter of 48 to 72 hours to the influence of growth hormone and thus might be used as an index of activity of growth hormone in rats.

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CALCIUM AND PHOSPHORUS IN THE BLOOD AND SKELETON OF THE ELASMOBRANCHII[1]

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ABSTRACT

Elasmobranchii, males as well as females, young and old, normally possessed high concentrations of calcium, 9.0 ± 3.8 meq./L in the serum. The level of calcium in the serum was independent of variations in the blood pCO_2, total CO_2, HCO_3^-, pH, total ionic strength, individual major electrolytes, total phosphate, inorganic phosphate, phospholipid, citric acid, urea, or alkaline phosphatase activity. The protein in the blood was relatively low in concentration, 4.8 ± 1.8 meq./L, had the chemical, electrophoretic, and ultracentrifugal properties of globulin, and showed the capacity to bind 4 mM of calcium per 100 gm. Approximately 75% of the calcium (7.5 ± 1 meq./L) was ultrafilterable; approximately 85% (6.2 ± 1.5 meq./L) of the calcium in the ultrafiltrate was physiologically active calcium ion as measured by perfusion of the heart of the shark; approximately 93% (7.0 ± 1.5 meq./L) was ionized as measured by the murexide method. The level of inorganic phosphorus was approximately 2.0 ± 0.8 meq./L; only 80%, presumably due to serum protein binding of phosphate, was ultrafilterable. The product of the ionized calcium and diffusible inorganic phosphate was twice that found in serum of bony vertebrates. The physical and chemical factors responsible for the high degree of supersaturation with respect to calcium phosphate in ultrafiltrates of serum require further investigation. Slices of cartilage from rachitic rats incubated in ultrafiltrates produced dense calcification. The calcium deposits in the cartilages of the elasmobranch consisted of an apatite mineral, having a Ca/P molar ratio of 1.52 .

SOME 400 million years ago, in the course of evolution, an ancient cartilaginous form gave rise to the Elasmobranchii of the present day. These fishes are among the most successful of modern vertebrate animals. The class Chondrichthyes, to which they belong, is important because the embryos of all higher animals, including man, must pass through a stage of development of shark-like branchial clefts and aortic arches.

Modern sharks, skates and rays possess enamel, dentine, and calcified cartilage, but no bone. An analysis of the composition of the blood and calcium deposits in the spine of these fishes reflects the course of evolution of the physiologic function of the skeleton. This communication will pre-

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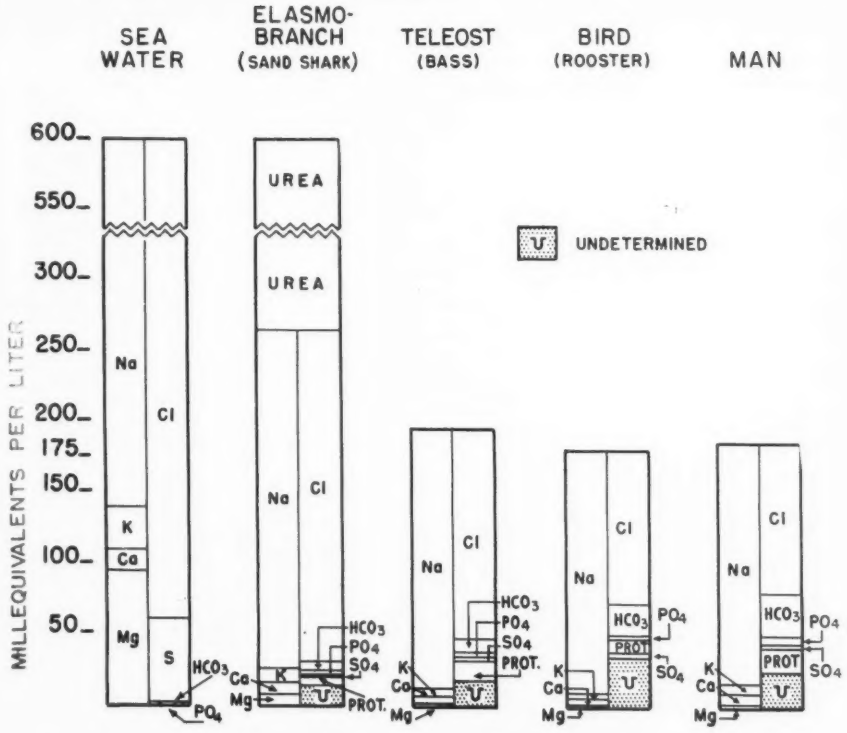

FIG. 1. Diagrammatic representation of the composition of the electrolyte in sea water and serum of an elasmobranch compared with serums of various higher vertebrates. Animals with bone tissue in the skeleton show lower levels total electrolyte, Ca, and Mg but higher levels of HCO_3, PO_4, and protein than the Elasmobranchii.

sent correlated observations of chemical, electrophoretic, ultracentrifugal components of serum, and various physical and chemical factors, influencing the calcium in blood and cartilage.

METHODS

The composition of the serums of the sand shark (*Carcharias littoralis*) was compared with that of Pacific Ocean sea water and serums of kelp bass, Black Minorca roosters, and normal adult human beings. Analyses were made for sodium, potassium, calcium, magnesium, chloride, sulphate, bicarbonate, inorganic phosphate, and plasma proteins by routine methods used in previous studies (1) and the results are summarized in Figure 1.

Whole serum and ultrafiltrates of serum from 75 sharks and rays were analyzed for calcium, inorganic phosphorus, protein, urea nitrogen, and other components. The serum proteins were examined by paper electrophoresis and the analytical ultracentrifuge. Calcium ion was measured by perfusion of the heart of the shark by means of a modification of the method of McLean and Hastings (2), and the murexide method of Rose (3).

The pH of cardiac blood was measured in five species, including the leopard shark *Triakus semifasiculatus*, which has the ability to ventilate the gills while resting on the bottom in a small aquarium. A Beckmann pH meter, model-GS, was employed with the glass and reference electrodes and the reference chamber mounted in a tank of sea water at a temperature of 16° C.

X-ray examination was made of the jaws and spine of one species of seven-gilled shark and eleven species of five-gilled sharks.

Histological sections were made of the spine of one seven-gilled shark and one five-gilled shark and stained with hematoxylin-eosin and azure for cartilage, Foot's method for collagen, and the von Kóssa technique for calcium salts.

The calcium deposits in three species of sharks and one teleost were examined by chemical, histochemical, and radiographic methods, and correlated with the results of high angle X-ray diffraction and infrared spectrography.

RESULTS

In order to interpret the results of analyses of serums of Elasmobranchii, it is necessary to compare the composition of the blood with the sea water in which they live. In a jawless cartilaginous vertebrate, such as the Hagfish, the plasma is isotonic with sea water, and the concentration of calcium is approximately the same in the internal and external environments of the organism. The ancestors of the elasmobranch fishes, however, evolved jaws of calcified cartilage, and the beginnings of ionic or osmotic independence from sea water.

Sea Water

The total cation concentration of filtered Pacific Ocean sea water is approximately 600 meq./L. The level of calcium is 20 meq./L, but the total phosphate, $HPO_4^=$ (valency 1.8 at pH 7.4) is only 0.0005 meq./L. The level of Mg was 95 meq./L; the Mg/Ca ratio was 5.5 compared with 0.35 in blood. (The levels of these constituents, as well as the others, are not appreciably different in the deep water of the Atlantic and other large oceans analyzed in modern times.) The level of calcium found in aquarium sea water was 19.0 meq./L; phosphorus, 0.001 meq./L. The average concentration of other components in sea water, compared with serum of vertebrate animals, was found to be as shown diagrammatically in Figure 1; the pH was approximately 7.9.

The extremely small concentration of phosphorus in sea water, compared with the high levels found in blood or tissue fluids of marine plants and animals, presents a unique situation. Living organisms are obviously able to concentrate phosphate ions from a very dilute solution. Radioisotope studies show that the phosphorous of the sea has an exceedingly high turnover rate (4). Phosphorus is also acquired in large amounts by marine animals by feeding on plants or other animal life.

The cell membranes of the gills and integumentary systems of marine elasmobranchs expend energy to sustain a concentration gradient for each ion separately between sea water and body fluids.

Blood

General Considerations.—In the Elasmobranchii, the hemocrit was 20% and the red blood count was only 200,000. As summarized in Figure 1, the total concentration of cation in the plasma was approximately 275 meq./L. It was characteristic of the elasmobranch, as found previously by many investigators, that the osmotic equivalent of 325 meq./L. of cation is retained in the blood plasma in the form of urea, trimethylamine oxide, and similar nitrogenous substance, and by this means, the organism maintains osmotic equilibrium between its tissue fluid, blood, and sea water. Aquatic, avian, or terrestrial animals having bone tissue have a blood total cation concentration of only approximately 150 meq./L and relatively insignificant amounts of urea. Various components of the blood in 8 small species and 9 large species of the Elasmobranchii were analyzed, as shown in Tables 1 to 3.

Urea.—The uremia varied from 1 to 2 gm.% urea, and other nitrogenous material. Nearly all of this was ultrafilterable. The osmotic effect was equal to 275 to 350 meq./L of cation, and served to maintain osmotic equilibrium between sea water and plasma of the shark.

Protein.—The level of protein in the serum was relatively low, varying from 3.0 to 9.7 meq./L (calculated as gm.% protein × van Slyke factor 2.43). This was precipitated by either Na_2SO_4 or NH_4SO_4 as globulin; there was only a small amount, less than 0.7 meq./L, of other protein remaining in solution and measurable by the biuret color reaction. The latter, however, was probably not albumin because it was not fast-moving by paper electrophoresis, or resolvable by ultracentrifugal sedimentation (11). The ultracentrifuge schlieren pattern revealed a mixture of at least four components, chiefly globulin and other uncharacterized protein (Fig. 2).

Inorganic Phosphorus.—The level of inorganic P (calculated as HPO_4^- at pH 7.4, valance 1.8) varied from 1.8 to 4.5 meq./L. These levels were found in serum freshly collected from sharks in an aquarium in the laboratory where the clot was removed by centrifugation without delay. Levels of 5.7 to 14.2 meq./L were found in specimens either hemolyzed or not separated early from the red cells; hydrolysis of organic phosphorus compounds probably occurred in the blood with levels of inorganic P above 4.0 meq./L.

Ultrafilterable Inorganic P.—The serum from 35 sharks and rays of 5 different small species were sacrificed in the laboratory where the clot was rapidly separated from the serum in order to minimize hemolysis, and obtain an ultrafiltrate rapidly without warming the blood to room temperature. While only 80 percent of the serum inorganic P was ultrafilterable, the Ca×P product of the ultrafiltrate was approximately twice that found in humans (Table 2).

TABLE 1. COMPOSITION OF THE PLASMA OF SMALL SPECIES OF ELASMOBRANCHII

Species	No. and sex	Ca. mg.%		Protein, Gms.%		Other	Inorganic P, mg.%	Total P, mg.%	Urea N, mg.%
		Total	UF	Total	Globulin				
Triakus semifasciculata	2 M	17.2±3.1	10.8±2.4	2.1±0.4	2.0±0.4	0.1±0.05	8.1±3.8	18.4±6.2	820±150
Triakus semifasciculata	2 F	14.9±2.9	10.0±3.0	1.2±0.2	1.0±0.1	0.2±0.04	13.0±3.1	26.0±8.4	1050±110
Cephaloscyllium uter	1 M	16.5	12.4	3.2	3.0	0.2	3.5	7.2	1050
Cephaloscyllium uter	1 F	20.9	16.0	3.1	3.0	0.1	4.64	14.72	1010
Rhinobatos productus	6 M	19.0±21	13.0±0.2	1.2±0.1	0.9±0.07	0.3±0.1	13.5±0.2	19.1±2.0	1020±201
Rhinobatos productus	4 F	20.4±2.1	12.1±3.1	1.3±0.1	1.1±0.1	0.2±0.1	11.0±3.0	19.9±4.0	1153±210
Heterodondous francisci	1 M	16.5	12.4	3.2	3.0	0.2	3.4	11.2	905
Heterodondous francisci	1 F	17.8	—	2.8	2.7	0.1	—	—	—
Notorynchus maculatum	1 M	12.67	11.79	2.0	1.8	0.2	8.0	18.4	1290
Notorynchus maculatum	1 F	11.79	11.01	1.7	1.5	0.2	8.2	17.3	1235
Platyrhinoidis triseriala	2 M	17.4±2.4	15.0±1.9	2.1±0.9	2.0±0.4	0.1±0.04	4.8±0.7	14.0±3.2	901±104

TABLE 2. ULTRAFILTERABLE INORGANIC P AND Ca×P PRODUCT OF ULTRAFILTRATES OF SERUM IN VARIOUS SPECIES OF SHARKS AND RAYS
(mg. %)

Species	No. fish	Total Ca	Inorganic P	UF Ca	UF Inorganic P	%, Inorganic P, filterable	UF Ca×P
latyrhinoidis triseriata	21	18.2±1.5	4.2±0.6	13.5±1.1	3.4±0.5	79±2	50±2
riakus semifasciata	5	22.1±2.1	5.20±0.8	19.18±1.5	3.9±0.2	75±2	75±2
rionace glauca	2	20.8±3.0	5.60±0.8	15.7±1.9	4.3±0.4	77±2	68±2
Heterodontus francisci	5	15.8±1.0	6.2±0.3	15.0±1.0	4.7±0.2	75±3	70±2
quatina californica	1	18.4	6.5	16.0	4.5	69	72
qualus suckleyi	1	17.4	6.0	16.0	4.7	78	75
ea Water	—	39.2±4.0	0.29±0.02	37.4±5.0	0.23±0.01	8±1	79±4
lan	—	10.3±1.0	3.8±0.1	4.8±0.3	3.8±0.1	100±5	18.2±2

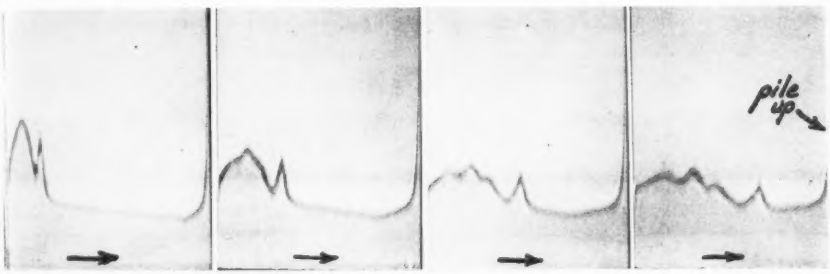

FIG. 2. Ultracentrifuge pattern of serum of *C. leucas*, showing 4 components, presumably globulins and other similar proteins (resolved 48 minutes after reaching a speed of approximately 170,000 g). The area of a peak is proportional to the concentration of each component.

TABLE 3. COMPOSITION OF THE SERUM IN LARGE SPECIES OF ELASMOBRANCHII

Species	No. and sex	Ca mg.%		Protein gm.%			Mg, mg.%	Inorganic P, mg.%	Total P mg.%	Urea N mg.%	Length	Embryos
		Total	UF	Total	Globulin	Other						
Carcharhinus leucas	2 F	18.0 ±0.4	15.1 ±2.0	2.4 ±0.2	2.2 ±0.2	0.2 ±0.04	5.8 ±1.0	10.6 ±0.1	19.5 ±2.4	918 ±340	5′3″ to 5′7″	
Carcharhinus littorales	1 F	18.8	15.0	2.3	2.2	0.1	5.0	14.4	27.2	1021	5′6″	+
Dasyatis lata	1 M	16.3	11.4	1.9	1.8	0.1	5.1	6.5	11.0	1140	—	−
Dasyatis lata	1 F	12.0	8.0	2.1	2.0	0.1	4.6	4.4	10.0	895	—	−
Eulamia obscuras	1 M	17.5	10.8	3.5	3.3	0.2	5.3	—	—	—	9′8″	−
Eulamia obscuras	1 M	16.2	14.2	2.4	2.2	0.2	5.3	9.2	14.0	910	10′1″	+
Eulamia milberta	2 M	18.7 ±4.0	14.9 ±0.8	2.4 ±0.1	2.3 ±0.1	0.1 ±0.01	4.1 ±0.04	11.0 ±4.1	26.0 ±6.2	998	5′3″ to 5′5″	−
Eulamia milberta	5 F	18.2 ±1.7	14.6 ±1.2	2.4 ±0.4	2.3 ±0.1	0.15 ±0.02	7.1 ±1.4	9.1 ±2.6	31.4 ±9.2	1004 ±10	5′4″ to 7′1″	+
Eulamia lamisorrah	1 F	21.1	16.7	3.3	3.0	0.3	5.9	18.8	50.0	—	5′5″	+
Carcharhinus auras	1 F	21.3	13.9	3.6	3.6	0.03	4.2	10.1	—	1420	9′8″	−
rionace auca	1 F	12.8	12.0	0.9	0.8	0.1	3.6	6.2	18.0	1049	7′6″	−

TABLE 4. THE LEVEL OF CITRATE ACID IN WHOLE SERUM ULTRAFILTRATES

Species	Citric acid, mg. %	
	Serum	Ultrafiltrate
C. leucas	1.6	1.6
C. littoralis	2.0	1.9
D. lata	0.4	0.4
D. lata	1.5	1.6

Total Phosphorus.—The level of total serum P varied from 3.1 to 28.8 meq./L. The higher levels were found in hemolyzed specimens collected at sea. The range in clear serums prepared in the laboratory was 3.1 to 12.7 meq./L.

Magnesium.—The level of magnesium in the serum varied from 2.0 to 5.8 meq./L, approximately 5% of the amount found in sea water. There was no correlation between the Mg content of sea water and serum and no apparent relationship between concentrations of Ca and Mg in either serum or ultrafiltrates of serums (Table 3).

Citric Acid.—The levels of citric acid in the serum varied from 0.4 to 1.9 mg.%. These levels are less than half those found in animals that have bone tissue in the skeleton (Table 4).

Phospholipid.—The levels of phospholipid (measured as lecithin) varied from 22 to 88 mg.% in whole serum (Table 5).

Alkaline Phosphatase.—The levels of alkaline phosphatase varied from 0.0 to 1.9 King-Armstrong units in 8 different species of sharks and rays (Table 6).

Total Calcium.—The level of calcium in the serum varied within a wide range of 5.9 to 12.3 meq./L. The level was uninfluenced by the age (as determined by length), sex, reproductive cycle, or species. (There were embryos in various stages of development in the uterus in 5 sharks.) There was no apparent relationship of calcium to the level of urea in the plasma.

Ultrafilterable Calcium.—The level of calcium in protein-free ultrafiltrates of the serum was approximately 75 to 90% of the total calcium; this did not appear to vary with wide variations in the level of urea.

Table 5. THE LEVEL OF PHOSPHOLIPID IN WHOLE SERUM AND ULTRAFILTRATES (mg. %)

Species	Phospholipid	
	Serum	Ultrafiltrate
E. milberti	82.0	39.0
E. obscuras	22	7.8
E. obscuras	27	5.2
E. menisorrah	88	48.0
P. glauca	30	30.0
E. milberti	66	21.0

TABLE 6. THE LEVEL OF ALKALINE PHOSPHASE IN THE SERUM IN SHARKS AND RAYS

Species	King-Armstrong units
E. obscuras	0.0
E. milberti	0.0
C. leucas	0.9
C. littoralis	1.0
P. glauca	0.4
E. menisorrah	0.0
C. lauras	1.4
D. lata, ♀	0.5
D. lata, ♂	1.9

The concentrations of citric acid and phospholipid were as low in ultrafiltrates as in whole serum, and thus could have been available for complexes with only a very small amount of calcium. The levels of Mg in ultrafiltrates were slightly lower than in whole serum.

Colloidal Calcium Phosphate.—Ultrafiltrates that were allowed to stand and warm to room temperature had a pH of 7.8; when the ultrafiltration of this fluid was repeated, there was lowering of the level of both calcium and phosphorus of only less than 0.3 mg.%. This may be regarded as evidence of formation *in vitro* of a small amount of colloidal calcium phosphate; there is no reason to assume that the phenomenon occurs in blood *in vivo*, except as a transient substance removed by macrophages, only to be returned in solution in lymph and tissue fluids as calcium and phosphate ions.

Partition of Calcium.—Samples of serum from *E. obscuras* were analyzed following ultracentrifugation in a partition cell, in a field of 70,000 G for 12 hours, and the results are shown in Figure 3. The composition of the middle two segments was the same. The top segment differed from them

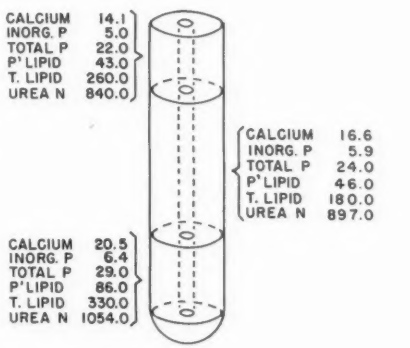

FIG. 3. Diagram showing the composition of the serum of *E. obscuras* and the concentration of electrolytes in 3 segments of a partition cell following ultracentrifugation. The non-protein bound calcium, as found in the upper segment, consists of approximately 80% of the concentration of the total calcium. The distribution of the protein is shown in Fig. 4.

WHOLE PLASMA: TOTAL CALCIUM = 16.2
TOTAL PROTEIN = 2.64 (Gms %), ALB.(9) = 0.16,
GLOB. = 2.48, INORG. P = 10.7, PHOSPHOLIPID = 66
TOTAL LIPID = 260, MAGNESIUM = 4.1, UREA = 1011,
ALK. PHOSPHATASE = 0.1 μ (Milligrams %)

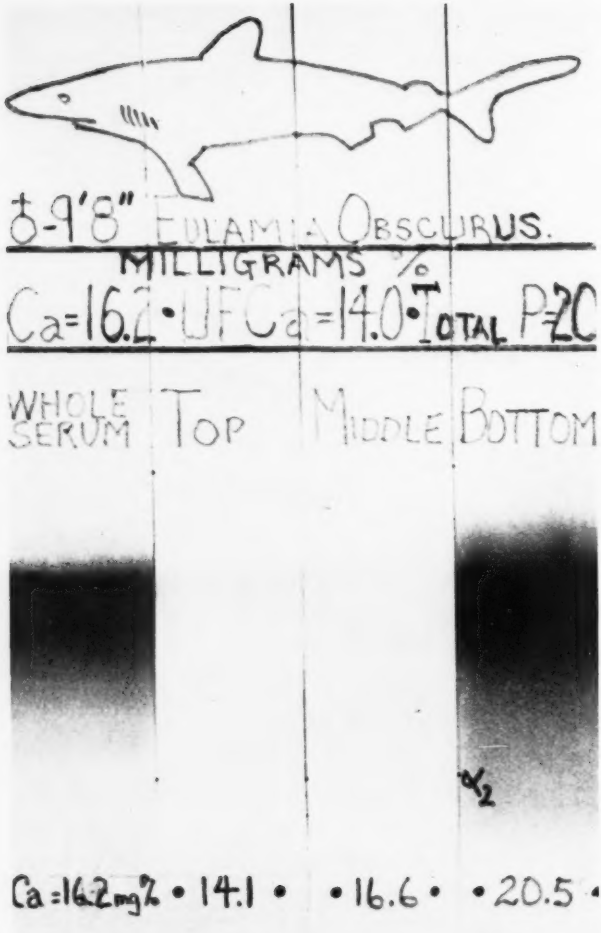

FIG. 4. Filter paper electropherograms of 10 lambda samples of whole serum and each of the 3 segments of the partition cell shown in Fig. 3. The bottom fraction contains various globulins and no fast-moving components or albumin. UFCa, an ultrafiltrate of the same serum, contained 14.0 mg. % calcium; the concentration of calcium bound to protein represented only approximately 20% of the concentration of the total calcium.

mainly in the higher concentration of lipid by floatation. The bottom segment differed from all others in the 4 to 6 mg.% higher concentration of calcium, and a slightly higher concentration of various other components. Ten lambda of serum from each segment were examined by paper electrophoresis (15 amperes for 12 hours; bromphenol stain) to determine the distribution of protein. As shown in Figure 4, the top and middle segments were protein free. The bottom fraction contained various globulins

TABLE 7. DISSOCIATION CONSTANTS FOR CALCIUM PROTEINATE FROM SERUM PROTEINS OF *Carcharinus leucas* DILUTED WITH Ca-FREE RINGER'S SOLUTION
(Globulin, 2.8; other proteins 0.1 Gms. %)

Dilution % serum	Total prot.		Total Ca mM/L	UF Ca obs. mM/L	CaProt mM/L	$Prot^-$ mM/L	pK_{CaProt}
	gm./100 cc	mM/L					
100	2.9	3.25	4.15	3.3	0.85	2.67	1.99
75	2.17	2.64	3.12	2.57	0.55	2.09	2.01
50	1.45	1.76	2.07	1.65	0.42	1.34	2.28
25	0.72	0.87	1.04	0.75	0.29	0.58	1.82

$$pK_{CaProt} = -\log \frac{[Ca^{++}] \times [Prot^-]}{[CaProt]}$$

[See McLean and Hastings, *J. Biol. Chem.* 108, Table IV, p. 293 (1935).]

but no albumin, and 6 mg.% higher concentration of calcium. Thus, the level of the non-protein bound calcium in the serum was as high as 85 to 90% of the total calcium. This unique relationship was attributed chiefly to the low concentration of plasma protein in the Elasmobranchii.

Calcium Proteinate.—Five samples of serum, 3 from *C. leucas* and 2 from *C. littoralis* were diluted in 4 stages with calcium-free Ringer's solution in order to determine the affinity or dissociation constants for serum proteinate of the shark. For application of the McLean-Hastings formula, the concentration of the ultrafilterable calcium was substituted for the concentration of calcium ion. A typical result, as shown in Table 7, demonstrated that the calcium dissociated from the protein in accordance with Mass Law relationships. The values for the mixture of globulins found in elasmobranch serum indicate that there is no essential difference in the calcium-binding properties of the globulins in the blood of shark and higher vertebrates.

The capacity of the Protein for Combination with Calcium.—Three samples of serum from *C. leucas* were diluted with solutions of various concentrations of $CaCl_2$ and NaCl in specified proportions, and the level of calcium was measured in ultrafiltrates of the mixtures. The results suggested that only a small amount of calcium could be added to the protein, and that the capacity for calcium binding was approximately 4.0 mM, Ca/100 gm. of the globulin in the blood of the shark.

Calcium Ion Concentration.—The level of ionized calcium in whole serum and ultrafiltrates of serum was estimated by means of perfusion of the heart of the shark, using a modification of the frog heart method (2). By comparison with standard solutions of calcium chloride in shark synthetic physiological media, the level of calcium ion, in either ultrafiltrates or whole serum, was estimated at 6.2 ± 1.5 meq./L. Urea was essential for normal function of the heart, but variations in the concentration from 1 to 1.5%, tested either in standard solutions or the serums, did not influ-

TABLE 8. BIOLOGIC ESTIMATION OF CALCIUM ION CONCENTRATION OF ULTRAFILTRATES OF SERUM BY PERFUSION OF THE HEART OF THE SHARK AND BY THE MUREXIDE METHOD

Solution	Ultrafilterable calcium meq./L	Calcium ion concentration meq./L	Percentage of ionic strength of sea water	% Ca^{++} in UF
Sea Water	20.3±3.0	—*	100	—
Physiologic Shark Sol.†	7.5±1.2	6.4±1.5	45‡	79
Serum, *P. triseriata*, s.h.	7.5±1.0	6.2±1.4	45‡	82
Serum, *P. triseriata*§	7.5±1.0	7.0±1.0	45‡	93
Serum, *H. sapiens*, f.h.	3.0±0.4	2.6±0.6	25†	83
Serum, *H. sapiens*§	3.0±0.4	2.8±0.5	25‡	90

* Responds to the maximum (10.0 meq./L) of the range of the heart.
† Includes 1.5% urea.
‡ Excludes urea, 1 to 2.0%
s.h. = shark heart; f.h. = frog heart.
§ Murexide determinations were made while bubbling oxygen containing 2.5% CO_2 through ultrafiltrates of shark serum and 5.0% CO_2 through ultrafiltrates of human serum; pH was 7.4 in each instance.

ence the ionization of calcium as it is observed by the perfusion method (Table 8).

pH of the Circulating Blood.—Samples of heparinized blood from 5 species of sharks were obtained to determine the possible relationship between the level of the non-protein-bound calcium and the pH. The results, as shown in Table 9, suggested that the pH of the blood of an elasmobranch fluctuates within a wide range of 7.12 to 7.69. The concentration of HCO_3^- buffer, the partial pressure of CO_2, and dissolved CO_2, were very low compared with man and other warm-blooded animals in which the pH of the

FIG. 5. Roentgenogram of the open jaws of *N. maculatum* showing teeth embedded in calcified cartilage. A cross section of spine of this seven-gilled primitive species (left) shows vertebrae consisting of *uncalcified* cartilage and notochordal tissue, and no mineral deposits of any kind.

TABLE 9. ACID-BASE BALANCE IN THE BLOOD OF ELASMOBRANCHII SPECIES

Species	pH	pCO_2 mm. Hg.	Dissolved CO_2 meq./L	HCO^3 meq./L	Total CO_2 meq./L	Total Ca meq./L	UF Ca meq./L
T. semifasciatus							
1st Day	7.40	8.88	0.28	5.09	5.37	10.3	8.4
2nd Day	7.12	12.95	0.39	3.15	3.54	7.6	5.0
7th Day							
Sample 1	7.13	10.14	0.29	3.00	3.29	6.7	5.0
Sample 2	7.57	8.24	0.248	4.69	5.17	7.2	5.6
Sample 3	7.50	9.50	0.287	4.60	4.94	10.4	8.0
N. maculatum ♂	7.18	12.60	0.35	5.0	5.35	6.3	5.8
N. maculatum ♀	7.30	13.09	0.395	6.07	6.46	5.9	5.0
Heterodondous	7.53	9.05	0.195	6.28	6.47	8.2	6.1
Cephaloscyllium	7.60	8.05	0.155	5.20	5.37	8.2	6.2
Aquarium water*	7.8	—	—	1.20	—	36.42	—

Total CO_2 was determined by Van Slyke manometric apparatus: pCO_2 and HCO_3 were calculated from the pH and total CO_2 at 15° C, ionic strength 0.30M, by the equation,

$$pH = 6.10 + \log \frac{[\text{total } CO_2] - 0.0301\ pCO_2}{0.0301\ pCO_2}.$$

* Aquarium conditions = T° 16° C.
Dissolved O_2 = 5.1 ppm, free or uncombined CO_2 = 6.16 ppm.

blood is regulated within a narrow range of 7.38 to 7.43. The amount of calcium bound to protein at 16° C appeared in the average sample to be greater at higher than at lower levels of the pH. The level of the total calcium did not vary with the level of the pH of the blood.

Temperature and pH During Ultrafiltration and Perfusion of the Heart.—The high level of diffusible calcium, as determined by ultrafiltration and calcium ion, as estimated by perfusion of the heart of the shark, could not be accounted for by effects of warming of the serum from 10 to 25 degrees or elevation of the pH from 7.4 to 7.8. Using a refrigerated centrifuge, ultrafiltration was carried out at 15 degrees C, a physiologic body temperature for Elasmobranchii. Refrigerated solutions, 15 to 20 degrees C, were used for perfusion of the heart. In general, lower temperatures reduced protein binding and raised level of ultrafilterable calcium. Loss of CO_2 and high pH increased protein binding and lowered the level of calcium in ultrafiltrates as much as 10 percent. At pH 7.4 and 25 degrees C, heart perfusion showed that the calcium ion concentration was less than 0.5 meq./L higher than at 15 degrees C.

The Equilibrium Between Intracellular and Extracellular Calcium.—Red cells of heparinized blood of *P. triseriata* were washed in 2.5% saline and 1% urea (a solution isotonic for shark blood), and analyzed for calcium and other inorganic ions. Another sample was washed in 2.5% KCl and 1% urea analyzed for sodium. The results demonstrate that the red cells (nucleated), and perhaps other cells of the elasmobranch, contain 2.0 meq./L or 4.0 mg.% of calcium, which is approximately 20% of the concentration of the diffusible (ultrafilterable) calcium of the plasma. The

TABLE 10. REGULATION OF DIFFUSION OF VARIOUS IONS. COMPOSITION OF PLASMA AND WASHED NUCLEATED RED BLOOD CELLS OF *Platyrhoidoidis triseriata*

(meq./L)

Component	Plasma	RBC.
Sodium	234.0	20.0
Potassium	11.4	175.2
Calcium	10.5	3.4
UF Ca	8.3	—
Magnesium	9.1	7.0
Chloride	208.0	160.4
Bicarbonate	3.2	—
Inorg. P.	3.8	13.4
Total P	27.0	77.4

FIG. 6. Roentgenogram of *R. productus*, a five-gilled ray showing calcified cartilaginous jaws and spine.

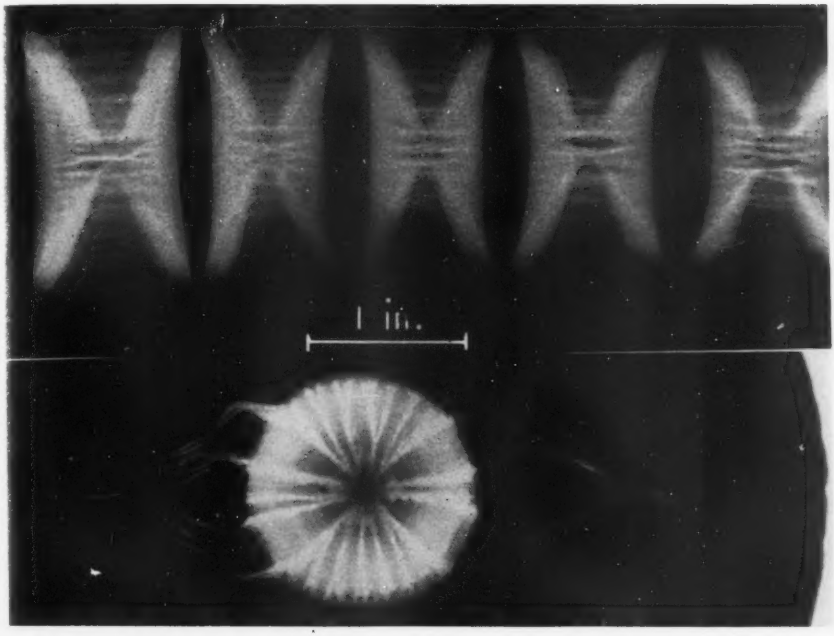

FIG. 7. Roentgenogram of spine of a mature female shark, *E. obscuras*, 10 ft., 1″ long, showing the detail of the pattern of the calcium deposits in the lumbar spine. Lateral view, above; cross section, below.

intracellular concentration of magnesium was even higher but the intracellular concentration of potassium was high and sodium low, as is generally the case in most higher vertebrates. These observations suggest that in the Elasmobranchii the cell membranes regulate the diffusion of Na^+ and K^+ ions separately from Ca^{++} and Mg^{++} and that this subclass is like the marine invertebrates that are adapted to the high concentration of the calcium and magnesium in the sea. Thus, the elasmobranch red cells have a form of cationic and osmotic regulation somewhat like that of the lower marine forms whose body fluids are in ionic equilibrium with sea water. Animals with cell membranes that regulate the diffusion of calcium and magnesium ions within a narrow range evolved an endoskeleton of bone tissue (Table 10).

Biological Solubility.—The minimum ion product, Ca×P, at which calcification will occur in mammalian cartilage, has been used as an index of biological solubility. Ultrafiltrates of human sera with a Ca×P product (expressed in mg.%), as low as 34, will produce calcification. Inasmuch as only 85% of the inorganic phosphate in the serum of Elasmobranchii is ultrafilterable (rather than 95% or more, as in man), and inasmuch as

FIG. 8. Roentgenograms of the jaws of a mature female shark, *T. fasciculata*, showing a healing fracture of the right mandible. The hook used to catch this shark is shown embedded in the calcified cartilage. Serial sections of the dorsal vertebrate (upper right, insert) demonstrate tectospondylic pattern of calcium deposit; note thin plates of mineral in cartilage supporting the neural canal and surfaces of cartilage.

heart perfusion determinations suggest that the largest part of the calcium (12.4 mg.%) is ionized, the minimum comparable Ca×P product is 63.24 ± 6.0.

Sera from 12 species were analyzed for magnesium and found to contain as much as 3× the concentration appearing in the blood of higher animals. Magnesium has been found to inhibit calcification *in vitro* in mammals (4). To investigate the possibility that the magnesium in the sera of Elasmo-

TABLE 11. CALCIFICATION OF RACHITIC RAT CARTILAGE SLICES IN ULTRAFILTRATES OF SERUM *in vitro*

Species	Ca × P product	Calcification
N. maculatum (♂)	96	4+
control + 1% urea	100	4+
N. maculatum (♀)	90	4+
control + 1% urea	50	4+
E. menisorrah	314	4+
control + 1% urea	200	4+

branchii might be a limiting factor in calcification in cartilaginous fishes, rachitic rat cartilage was incubated in ultrafiltrates from 3 species (Table 11). Standard solutions of the composition of those of Yendt, *et al.* (4), with the addition of 1% urea, were used for controls. Neither the magnesium in the experimental sera nor the urea in the controls inhibited calcification of rachitic cartilage slices.

Table 12 summarizes the results of experiments designed after the method of Nordin (6) in which the pH was measured at the point of precipitation of calcium phosphate salts from water, physiologic shark salt solution, and ultrafiltrates of serum from *T. semifasciculata*, titrated with 0.1N NaOH. The ultrafiltrate was examined before and after dilution with

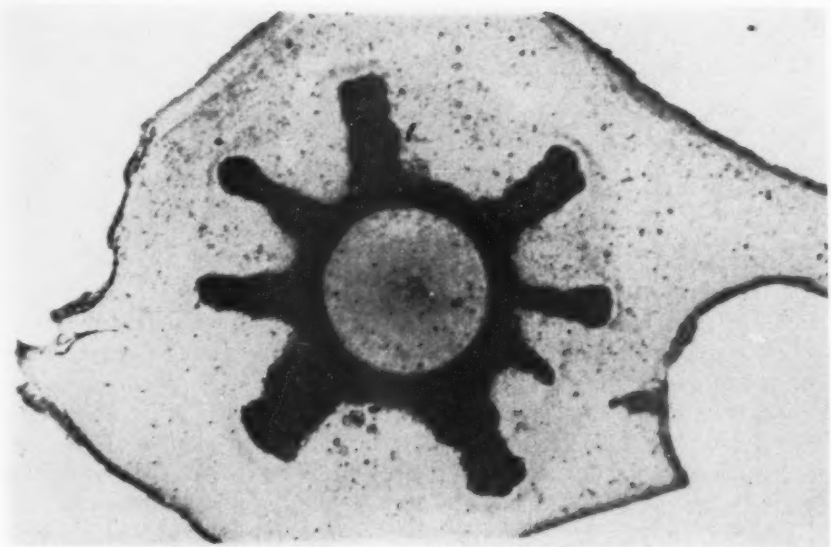

FIG. 9. Histologic section of the caudal vertebra of the same shark as shown in Fig. 8. Hematoxylin-eosin- and azure (left) showing a cross section of the structure of the core of calcified cartilage.

TABLE 12. RELATIONSHIP BETWEEN pH AND PRECIPITATION OF CALCIUM PHOSPHATE FROM WATER, PHYSIOLOGIC SHARK SALT SOLUTIONS, AND ULTRAFILTRATES OF SERUM (CONCENTRATION = 15 MG.% CA AND 5 MG.%) INORGANIC PHOSPHORUS

Solution	pH of solution*	pH of precipitation†
A Water	6.85 ± .1‡	7.48 ± .02
B Physiologic Shark Salt Sol.	7.41 ± 1	7.61 ± .03
C Ultrafiltrate	7.75 ± 1	8.01 ± .06
D Ultrafiltrate 1:1 Sol. A	7.40 ± 1	7.55 ± .03
E Ultrafiltrate 1:2 Sol. A	7.39 ± .1	7.45 ± .06
F Ultrafiltrate 1:3 Sol. A	7.40 ± .1	7.57 ± .05

* In room air at 10° C.
† Precipitate obtained by titration with 0.1N NaOH; pH at point of redissolving of precipitate determined with 0.1N HCl.
‡ Acidified with HCl to retain the calcium in solution.

water solutions containing standard of 15 mg.% Ca and 5 mg.% inorganic phosphate in the form of $CaCl_2$ and Na_2HPO_4, sustained by an initial pH of 7.0. The undiluted ultrafiltrate differed from the synthetic solutions and diluted ultrafiltrate in that the high pH of 8.01 was required to produce gross precipitation of calcium; at 10 to 20 degrees C, 7.45 to 7.61 was the range of the pH at which control solutions produced this reaction. Thus, the shark possessed some special factor that is responsible for supersaturation of the serum with respect to calcium and inorganic phosphate ions; this deserves intensive investigation.

Skeleton

The Calcium in Cartilage.—All species of five-gilled sharks and rays had calcium deposits in their crania, jaws and vertebra. Calcification occurred

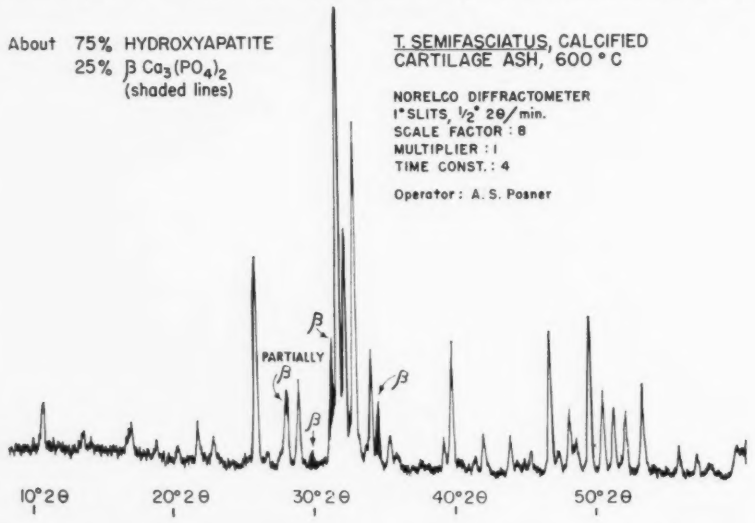

FIG. 10. X-ray diffractogram of the calcium deposits in the vertebral bodies shown in Fig. 8. The triplet peaks above 30° 2θ are the same as those obtained from bone ash.

n the surfaces of the vertebra in some parts of the spine and in the center f the vertebral body in other parts in a pattern that is characteristic of ach species. The calcium deposits were found in masses or plates in a pecial form of matrix. The uncalcified parts were fibro-cartilage or hyaline artilage containing very little collagen. The calcified matrix was very ensely packed with collagen, and stained metachromatic with toluidin lue and positive with periodic acid and Schiff reagents; the cells enclosed i it had thin capsules and contained alkaline phosphatase.

N. maculatus, a primitive seven-gilled shark (Table 3), found off shores f California, was of particular interest because it possessed jaws of calcified artilage but no calcification in the vertebra. The vertebral bodies consisted f a core of loose gelatinous notochordal tissue within a shell of fibrocartiage and fibrous tissue. There was hyaline cartilage with basophilic matrix nd strips of fibrocartilage; the calcium bearing metachromatic, collagenich matrix was absent in the spine.

The Calcium Salt.—The calcium deposits in the spine of three representative species of five-gilled sharks had an ash weight of only 20% of the wet weight of the vertebra; the ratio of Ca/P on a molar basis was 1.52. For purposes of comparison, the Ca/P ratio of bone mineral of the teleost, *Sphyraena barracuda*, was 1.62. X-ray diffractograms and infrared spectrographic analysis of samples ashed at 600 degrees showed 25% β-tricalcium phosphate and 75% hydroxyapatite. The X-ray diffraction pattern of the wet material, as well as the ash, was that of an apatite similar, if not identical, to the mineral of bone (Fig. 10). Further studies of the crystal structure, size, and orientation of the calcium deposits in the cartilage of the shark, as in the bones of higher vertebrates, are necessary to determine whether the mineral is hydroxyapatite, carbonate apatite, hydrated tricalcium phosphate, calcium-deficient hydroxyapatite, or octocalcium phosphate.

DISCUSSION

The circumstances associated with calcium in the blood of the Elasmobranchii have received relatively little attention. Teleost fishes, in comparison, have been investigated more thoroughly, probably because of the marked elevation of the level of serum calcium (5.5 ± 1.0 to 10 ± 4 meq./L) that was found in females during reproduction. The elasmobranch fishes were found to have comparable levels in both females and males (7). Similar observations were recorded later without comment by others (8, 9, 19). Our data presented above demonstrated that high levels of calcium are probably characteristic of the Elasmobranchii of all species, both sexes, mature and immature, gravid and non-gravid. Observations presented in a previous report (11) revealed that teleosts, as well as higher vertebrates of oviparous and some ovoviviparous species developed hypercalcemia only in females during the reproductive cycle; the calcium that was added to the

FIG. 11. X-ray diffraction patterns of the calcium deposits in wet vertebra of the elasmobranch, *P. glauca* (right), and the teleost, *S. barracuda* (left). Note the identical x-ray diffraction pattern of the cartilage and bone minerals. (CuK radiation and camera with R = 28.7 mm.; operator—F. Poutard, Ph.D.)

serum was bound to protein in transport from the liver to the yolk sac and always associated with the deposition of egg yolk.

The level of protein in the serum was relatively low in all species of elasmobranchs. The chief component was globulin with either no albumin (12) or some other atypical non-fast moving electrophoretic component (13, 14, 15). Our observations corroborate these results in that there were only small amounts of protein in solution following Na_2SO_4 precipitation, and no fast-moving components. Ultracentrifugal analysis showed a schlieren pattern of four separate components of almost equal concentration. It may be assumed that the Elasmobranchii have not evolved a protein comparable to the serum albumin of higher vertebrates.

The acid base balance in the blood has received considerable attention in connection with studies dealing with peculiar respiratory and excretory physiology of the elasmobranch fishes. Single determinations of the pH of the blood of individuals of different species have been recorded and found to fall within a wide range of 7.20 to 7.63 (8). Others found 7.82 in arterial blood and 7.67 in venous blood (16); 7.49 (17), 7.3 to 7.5 (18), or 7.55 (19) in mixed blood. Previously published data also revealed a low bicarbonate buffer capacity, 5.56–11.5 meq./L (8), or 4–5 meq./L (20). Our observa-

ions confirm these results, and suggest, based on serial observations on *T. semifasciatus*, as well as single observations on various species, that (a) the HCO_3^-, total CO_2 and pCO_2 are normally low, and (b) the pH of the blood of the elasmobranch normally fluctuates within a wider range than found in higher vertebrates having a high concentration of blood bicarbonate. The level of calcium in the blood was probably not influenced by factors associated with acid-base balance, although it was likely that the level of the ultrafilterable fraction was slightly higher when the pH was low.

The level of inorganic phosphorus was also more variable than that generally found in higher vertebrates, 80 to 85%,

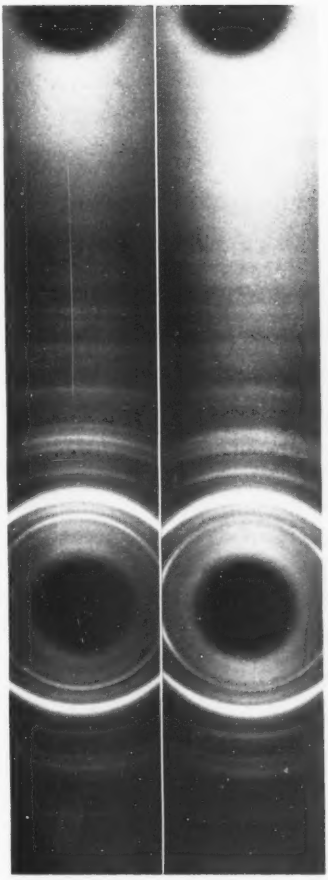

FIG. 12. X-ray diffraction patterns of the calcified vertebra of the shark, *P. glauca* (right) and synthetic apatite (left). The diffraction pattern of cartilage shows evidence of preferential orientation of the crystallites; otherwise, there is remarkable similarity of the shark mineral and *in vitro* bone mineral. (CuK radiation and camera with r = 28.7 mm.—prepared by D. McConnell, Ph.D.)

rather than 95 to 100%, as in man, was ultrafilterable. It is unlikely that colloidal calcium phosphate can account for non-ultrafilterable phosphate because it did not sediment by ultracentrifugation and there was no partition of calcium apart from protein binding. It is very likely that an equilibrium exists between free and non-diffusible inorganic phosphate ions (21), presumably due to protein binding of inorganic phosphate (22, 23, 24).

The Ca×P product of ultrafiltrates of serum was 2 to 3× higher in elasmobranchs than bony vertebrates; the factor responsible for supersaturation of these solutions with respect to calcium phosphate remains to be elucidated by further experiments.

The high proportion of calcium in ultrafiltrates and ultracentrifugal supernatant fluids may be attributed to the low level of serum protein (consisting almost entirely of globulins). In accordance with the mass law,

using the McLean and Hastings nomogram (25), the calcium ion concentration in a serum containing a total calcium ion concentration of 8.9 ± 09 meq./L, may be estimated at 5.8 meq./L. By means of perfusion of the heart of the shark, the level of calcium ion was found to measure 6.2 meq./L. The ultrafilterable calcium was 7.5 ± 1.0 meq./L, of which 82%, as seen by the perfusion method, and 93% by murexide method, is ionized and non-protein bound. While 1 to 2% urea is a constituent of the blood that is essential for normal function of the heart of the shark (26, 27, 28), it had no material effect on the ionization of calcium or precipitation of calcium phosphate from synthetic shark physiologic salt solutions (29).

The ionic strength[2] of ultrafiltrate of elasmobranch serum was approximately 0.3 M. The $pKs \cdot p \cdot Ca_3(PO_4)_2$ has been calculated in salt solutions of ionic strengths of 0.146 M to 0.184 M, the range of human serum, and found decreasing with increasing ionic strength in accordance with the Debye and Hückel theory of strong electrolytes (30). Similar data are not available for ionic strengths of blood of elasmobranchii and lower vertebrates, blood, or sea water, in which it is possible that the activity coefficient and the $pKs \cdot p \cdot Ca_3(PO_4)_2$ may again rise if these solutions follow the curves drawn for the Debye and Hückel theory.

Rachitic rat cartilage incubated in solutions of .3 M ionic strength with $Ca \times P$ products of 50 produced 4+ calcification (31), the same as we have described in this report with ultrafiltrate of shark serum. At ionic strengths, 0.415 M calcification was inhibited (31); these conditions can be found only in marine invertebrates and protochordates and it would be of great interest to determine whether calcification of rachitic cartilage would occur in haemolymph from these animals.

The concentration of calcium ions in the blood of Elasmobranchii is slightly more than twice that found in bony vertebrates. One explanation for the high tolerance of lower marine vertebrates to relatively high levels of calcium ions in blood and sea water may be that the effects of calcium ions are not due to single ions but to ion ratios involving Na^{++}, K^{++}, Mg^{++}, OH^- and H^+ in the solution. While this cannot be expressed in mathematical terms, it is as a pharmacologically meaningful and useful concept. The concentrations of ions in the expression:

$$\frac{Na^+ \times Ca^{++} \times OH^-}{K^+ \times Mg^{++} \times H^+} \propto \text{myocardial irritability}$$

in the elasmobranch are roughly twice those found in bony vertebrates,

[2] Ionic strength, designated by the letter "μ," in the formula of Lewis and Randall, $\mu = \frac{1}{2}\Sigma c_1 z_1^2$ was calculated for phosphate-free physiologic shark salt solutions and found to be 0.2997M; for artificial sea water, 0.6842M; for mammalian Ringer's solution, 0.1623M. Phosphate was omitted from these solutions because of precipitation of calcium phosphate in water solutions lacking in organic substances with the effect of a crystal formation inhibitor.

TABLE 13. RELATIONSHIP BETWEEN AQUATIC HABITUS, ION GRADIENTS, AND SKELETAL TISSUE OF VERTEBRATE ANIMALS

(meq./L)

Component	Sea water	Cyclostome*	Elasmobranch	Teleost	Man
Cation	600	619	260 + urea	194	155
Na	509	558	235	180	140
K	30	10	10	5	5
Ca	20	20	10	6	5
Mg	95	39	6	3	2
Cl^-	540	576	230	147	103
HCO_3^-	2	4	5	10	27
Inorg. P*	0	3	3	3	3
$SO_4^=$	60	13	1	1	1
Protein†	—	—	5	7	17
Skeleton		Uncalcified cartilage	Calcified cartilage	Cartilage and bone	Cartilage and bone
Percent Reduction of Blood Electrolyte	—	0	56	68	75
Percent Reduction of Blood Calcium	—	0	50	75	75

* Based on mean valency of 1.8, 80% HPO_4 and 20% H_2PO_4.
† Calculated by Van Slyke Method, Gms.% Protein × factor of 2.43.

and one-half those found in cyclostome. Under these circumstances, the ratio of the ions to each other is the same in all species. The *sensitivity* or capacity of the myocardium to respond to calcium ion thus could be influenced by the pattern of the composition of plasma ions; with due regard for the physical-chemical laws of solutions, each ion seems to be regulated separately by the cell membranes.

The calcium deposits in the vertebrae of all of the five-gilled species appeared to be determined by the structure of the cartilage. In the course of evolution, the uncalcified vertebrae probably preceded the calcified, the cyclospondylic preceded the tectospondylic; the astrospondylic was the latest development (32, 33). Calcification was present in the jaws but absent in the vertebrae of the seven-gilled shark, *N. maculatus* (34). Our observations confirmed these findings, revealed that the composition of the blood was the same as that of other Elasmobranchii, and suggested that a calcified endoskeleton evolved with an increase in the density of the collagen and other local factors of the matrix of the cartilage cells. Examination of mineral in the skeletons of sharks, lungfish, and various bony vertebrates by X-ray diffraction showed apatite patterns in all instances (35): Our observations corroborate this finding and add the conclusion that the chemical and crystal structure of the apatite is the same in the Elasmobranchii as found in the skeleton of bony fishes and higher vertebrates.

The implications of the foregoing work on the subject of evolution is of great interest. In Table 13 we have combined the data in the literature on the marine cyclostomes (36, 37), [a lower vertebrate having no calcified cartilage anywhere in the skeleton] with our data on the Elasmobranchii

and higher vertebrates. This reveals that the percent reduction in the total electrolyte and in the total calcium in the blood was remarkably parallel—0% by animals with uncalcified cartilage, 50% by elasmobranchs with calcified cartilage, and 75% by vertebrates having bone in the skeleton. The high level of calcium in the blood of the lower vertebrates was associated with a correspondingly high concentration of the total electrolyte in the blood, but this did not reduce activity coefficient of calcium to appreciably lower levels in elasmobranchs than in higher vertebrates. The results of perfusion of the heart of the shark and analyses by the murexide method suggested that the elasmobranch is adapted to high concentrations of calcium ion in the blood (29). One of the specific functions of calcium ions in lower animals is concerned with the lowering of the permeability of the cell membranes to water. In higher vertebrates, calcium and other cations to be found in sea water appear to be stored in the skeleton to protect the internal environment against changes in the external environment; bone as a tissue seems to have been evolved by the vertebrates as an integral part of their achievement of osmotic independence.

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INDUCTION OF MAMMARY SECRETION IN PREGNANT RATS AND RABBITS BY HYDROCORTISONE ACETATE[1]

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ABSTRACT

A dose of 2 mg. of hydrocortisone acetate (HCA) per day, injected during the 5–9th or 9–13th days of gestation in rats initiated mammary secretion on the 10th or 14th days, respectively. This treatment had no effect on duration of pregnancy or number of viable young at birth. The same dose of HCA also initiated mammary secretion in rats when administered during the 9–13th days of pseudopregnancy. All other hormones including prolactin, oxytocin, ACTH, and combinations of prolactin and ACTH or prolactin and oxytocin were ineffective at the dose levels used. A dose of 15 mg. HCA per day initiated copious milk secretion in rabbits on the 20th or 21st day of pregnancy when administered during the 16–19th days of pregnancy. A dose of 1 I.U. oxytocin given 3 times daily was ineffective in initiating lactation in the rabbits. It is suggested that the levels of biologically active adrenal glucocorticoids may not be sufficiently high during pregnancy and therefore may be partially responsible for the absence of mammary secretion during this state.

MAMMARY growth takes place during pregnancy but lactation is usually not initiated until about the time of parturition. This has been explained on the basis of the interaction between the pituitary, ovarian steroids and the mammary gland (1). Both *in vivo* and *in vitro* studies have shown that adrenal glucocorticoids are essential for initiation and maintenance of mammary secretion in rats, mice and guinea pigs (1–3), and there is evidence that adrenal cortical function is increased following parturition (4). Cortisone (5) and hydrocortisone acetate injections (6) have also increased established milk yields in the post-partum rat.

Pregnancy or administration of estrogens has been reported to induce important alterations in adrenal steroid metabolism. Although estrogen stimulates ACTH secretion (7), it has been reported (a) to decrease corticosterone or cortisol synthesis in the rat, guinea pig, dog and man (8–11), (b) to increase the corticosteroid-binding protein (transcortin) levels in the blood and transcortin-binding of corticosterone or cortisol, and not to

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ncrease non-protein bound cortisol (11–13), and (c) fail to reduce signifi-antly the eosinophil count in the rat, guinea pig and man (14–16, 11). Several workers have suggested that only non-protein bound glucocorti-oids are biologically active (10–13). The effects of pregnancy on adrenal teroid metabolism appear to be comparable to those produced by estrogen 11, 13, 17). Thus pregnant rats and women apparently show increased re-istance to the physiological and metabolic effects of cortisone (18, 16). These observations suggest that the levels of biologically active adrenal lucocorticoids may not be sufficiently high during pregnancy and therefore nay be partially responsible for the absence of lactation during this state. The present study was undertaken to investigate this possibility in the at and rabbit.

METHODS

Mature virgin female rats (Carworth, CFN strain) weighing 200–225 gm. each, were housed in an air-conditioned room. Food and water were available at all times. Two male rats were placed with five females in each breeding cage, and the females were checked twice daily for evidences of copulation. When a copulation plug was found, the female was removed and placed in an individual cage. This was considered the first day of pregnancy. All injections were made subcutaneously in 0.1 ml. volumes. Hydrocortisone acetate (HCA) was supplied as a saline suspension (25 mg./ml.), ACTH (40 U.S.P. units/ml.) in a gel preparation and oxytocin (280 I.U./ml.) in saline. Prolactin (15 I.U./mg.) was dissolved in distilled water.[3]

The rats in Groups 1–10 were treated daily with hormones on the 9–13th days of pregnancy as shown in Table 1. Some of the rats became pseudopregnant (Groups 11 and 12) and these were given either saline or HCA. Groups 13 and 14 were treated on the 5–9th days rather than on the 9–13th days of pregnancy. Biopsies were performed under ether anesthesia on the inguinal mammary glands of all rats of Groups 1–12 on day 14 of pregnancy or pseudopregnancy. In Groups 13 and 14, biopsies were performed on day 10 of pregnancy. The methods used for histological preparation of the mmamary tissues have been described elsewhere (19). The rats were not disturbed during the remainder of pregnancy, and the day of parturition and the number of young born were recorded.

Female New Zealand White rabbits, weighing 3.5–4.5 kg. each, were treated daily during the 16–19th days of pregnancy (Groups 15–17). On days 20 and 21, attempts were made to express milk from the nipples. A few rabbits were killed at this time and their mammary glands were exposed for photography. Most rabbits were permitted to continue their pregnancies to term, and the day of parturition in each case was recorded.

RESULTS

Only 2 of 10 rats showed microscopic evidence of mammary secretion when injected daily with 1 mg. of HCA during the 9–13th days of pregnancy (Group 3). However, 2 mg. of HCA per day initiated mammary secretion in all 10 animals (Group 4, Figs. 1 and 2). HCA was effective in initiating

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TABLE 1. EFFECTS OF HORMONE TREATMENTS ON INITIATION OF MAMMARY SECRETION IN PREGNANT RATS AND RABBITS

Group	No. of animals	Treatment*		No. of animals with secretion
		RATS		
		9–13th Days of Pregnancy		
1	10	Controls, saline	1×d	0
2	10	0.5 mg. HCA	1×d	0
3	10	1 mg. HCA	1×d	2
4	10	2 mg. HCA	1×d	10
5	5	1 mg. Prol.	2×d	0
6	5	2 mg. Prol.	2×d	0
7	4	1 I.U. OX	2×d	0
8	5	1 mg. Prol+1 I.U. OX	2×d	0
9	4	1 U.S.P. Unit ACTH	2×d	0
10	5	1 mg. Prol+1 U.S.P. Unit ACTH	2×d	0
11	4†	Controls, saline	1×d	0
12	4†	2 mg. HCA	1×d	4
		5–9th Days of Pregnancy		
13	4	Controls, saline	1×d	0
14	4	2 mg. HCA	1×d	4
		RABBITS		
		16th–19th Days of Pregnancy		
15	5	Controls, saline	1×d	0
16	3	1 I.U. OX	3×d	0
17	8	15 mg. HCA	1×d	7

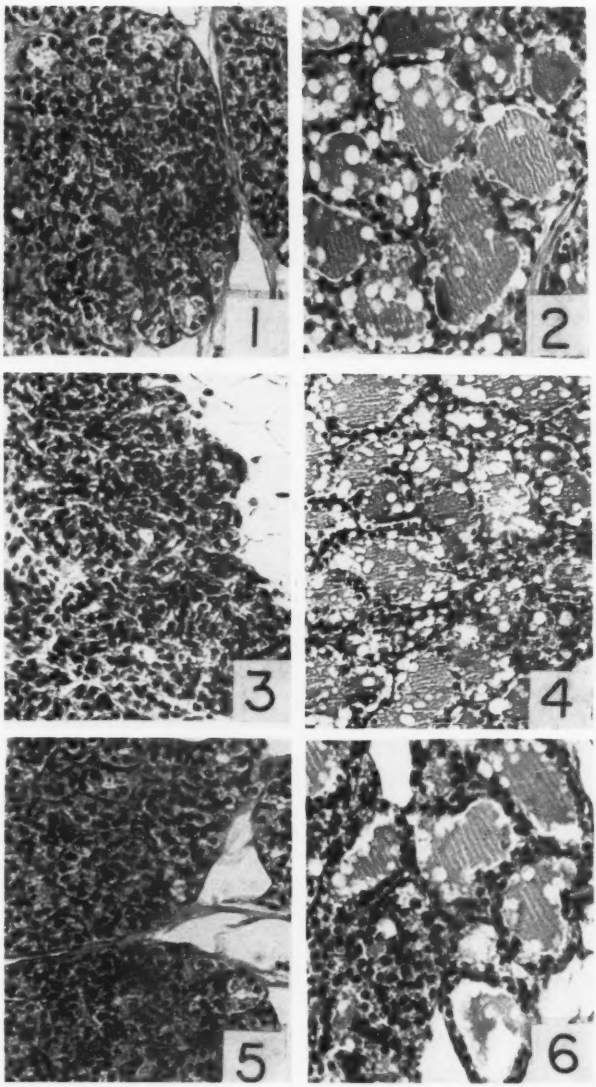
* HCA = Hydrocortisone Acetate, Prol. = Prolactin (15 I.U./mg.), OX = oxytocin, d = daily.
† Pseudopregnant rats.

mammary secretion as early as the 10th day of pregnancy when injected during the 5–9th days after mating (Groups 13 and 14, Figs. 3 and 4). The pseudopregnant rats (Groups 11 and 12, Figs. 5 and 6) also showed mammary secretion when injected with 2 mg. HCA per day. The following treatments were ineffective in pregnant rats: saline (Group 1); 0.5 mg. HCA per day (Group 2); 1 or 2 mg. prolactin twice daily (Groups 5 and 6); 1 I.U. oxytocin twice daily (Group 7); 1 mg. prolactin and 1 I.U. oxytocin twice daily (Group 8); 1 U.S.P. unit ACTH (Group 9); 1 mg. prolactin and 1 U.S.P. unit ACTH twice daily (Group 10). None of these treatments had any effect on duration of pregnancy or number of viable young at parturition.

In rabbits, saline or oxytocin injections during the 16–19th days of pregnancy did not initiate milk secretion (Groups 15 and 16), while 15 mg. HCA per day induced copious milk flow (Group 17, Fig. 7). Considerable milk could be expressed from the nipples on days 20 or 21 of gestation in 7 of 8 rabbits. Treatment with HCA in the rabbits had no effect on duration of pregnancy or on the condition of the young at birth.

DISCUSSION

The alveoli of pregnant rats usually remain tightly closed with little evidence of secretory activity until about the 19th day (20), while in rabbits mammary secretion does not appear until about the 29th day of gestation (21). Systemic injections of prolactin extracts have failed to



FIGS. 1–6. Photomicrographs of representative sections of mammary glands. ×150.

1. From rat treated with saline during 9–13th days of pregnancy and biopsied on 14th day.
2. Same as 1, but treated with 2 mg. HCA per day.
3. From rat treated with saline during 5–9th days of pregnancy and biopsied on 10th day.
4. Same as 3, but treated with 2 mg. HCA per day.
5. From rat treated with saline during 9–13th days of pseudopregnancy and biopsied on 14th day.
6. Same as 5, but treated with 2 mg. HCA per day.

FIG. 7. Mammary glands from rabbits treated during 16–19th days and killed on 20th day of pregnancy. Control animal above was injected with saline and shows no secretory activity on 20th day of pregnancy. The rabbit below was treated with 15 mg. HCA daily and shows abundant lactation. Normally developing fetuses were found in the uterine horns of both rabbits.

initiate mammary secretion in pregnant rats, rabbits, guinea pigs and goats unless death of the fetuses or abortion was first induced (22). The prolactin preparations used were not highly purified. A localized lactation was initiated in pregnant and pseudopregnant rabbits by intraductal administration of prolactin, but similar treatments in pseudopregnant rabbits with "adrenal cortical extract," STH, TSH, ACTH, FSH, ICSH, oxytocin or vasopressin were ineffective (22, 23). In virgin and estrogen-primed rats, cortisone and HCA have induced mammary secretion and increased pituitary prolactin content (1). The present experiments demonstrate that HCA alone can initiate mammary secretion in pregnant rats and rabbits without any discernible effect on the duration of pregnancy or on the condition or number of young born. In pseudopregnant rats both prolactin as well as adrenal cortical extracts have been reported to be necessary to initiate mammary secretion (24), but the present study shows that HCA alone can initiate mammary secretion in pseudopregnant rats. It has been observed that adrenalectomy during pregnancy in the rat does not prevent initiation of a slight, transient lactation after parturition, but this may be due to the presence of placental and fetal adrenal factors (1).

The inability of oxytocin to initiate mammary secretion in pregnant rats and rabbits is in agreement with the previous work showing that this hormone does not initiate mammary secretion in these species (25). The dose of ACTH employed did not initiate mammary secretion in pregnant rats, although HCA was effective. This may be due to diminished responsiveness of the adrenal cortex to ACTH stimulation, as has been previously observed during pregnancy or after estrogen administration (8, 9, 16, 26). Prolactin alone or in combination with ACTH also did not initiate mammary secretion in the pregnant rat. This may be due to inability of prolactin to act on the mammary gland in the absence of sufficiently high levels of biologically active glucocorticoids, since increased adrenal cortical secretion as well as prolactin are necessary to initiate mammary secretion in the rat (1, 27). It is also possible that the dose of ACTH used may have been too low to stimulate the adrenals adequately.

The pregnant rat and rabbit appear to be relatively resistant to several of the biological effects of high doses of cortisone (18). A dose of 3 mg. per day of cortisone given during the 6–19th days of pregnancy to rats was previously reported to have no effect on the course of pregnancy or average weight and number of young born alive (28). Doses of cortisone as high as 20 or 25 mg. per day also had no significant effect on the course of pregnancy, litter size or survival rate in the rat, although the average weight of the young at birth was reduced (18, 29). Injections of 10 mg. per day of cortisone, given subcutaneously during the 13–18th days of pregnancy had no effect on the course of pregnancy in rabbits, while abortion and mascerated fetuses were observed following intramuscular injections of 20 mg. per day of cortisone (30). Subcutaneous injections of the higher dose were not tried in these rabbits.

The present results suggest that during pregnancy a decreased synthesis and/or increased protein binding of adrenal glucocorticoids may prevent them from reaching levels sufficient to stimulate mammary secretion. This may be due to the high levels of estrogen present during pregnancy, although progesterone may also be involved (8). With the decrease in circulating levels of ovarian steroids at parturition, the availability of biologically active glucocorticoids may rise (4) and this together with the increase in prolactin secretion and removal of the estrogen-progesterone stimulus to mammary growth (1) would permit the onset of mammary secretion.

Addendum

We recently injected several doses of corticosterone, the major glucocorticoid of the rat adrenal, into pregnant rats (unpublished). A dose of 8 mg. daily during the 9–13th days of gestation failed to initiate mammary secretion by the 14th day in any of 6 rats; a dose of 12 mg. daily produced mammary secretion in 2 out of 9 rats; and 16 mg. daily induced mammary secretion in 5 out of 5 rats. None of the doses of corticosterone interfered

with the course of gestation. This indicates that corticosterone is much less active (about 1/8) than HCA in producing mammary secretion in pregnant rats. The relatively high dose of 5 U.S.P. units of ACTH, injected twice daily during the same period of pregnancy, failed to induce mammary secretion in any of 5 rats. In an independent study, Nandi and Bern (*General and Comparative Endocrinology*, In press) recently concluded that inadequate secretion of glucocorticoids is the limiting factor in initiating mammary secretion during pregnancy in an inbred strain of mice.

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STIMULATION OF ANTERIOR PITUITARY AND CEREBRAL CORTICAL GLUCOSE OXIDATION BY NEUROHUMORAL AGENTS

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ABSTRACT

Epinephrine, norepinephrine and serotonin stimulate *in vitro* glucose-1-C^{14} oxidation to $C^{14}O_2$ by anterior and posterior pituitary slices, and cerebral cortical fragments. The effect was demonstrable with 15-minute incubations. It was not observed with testis or liver. Glucose-6-C^{14} and pyruvate-2-C^{14} oxidation were not influenced by these agents. No stimulation of glucose uptake by epinephrine or serotonin could be demonstrated. Ergotamine, dibenzyline and DCI did not block the epinephrine effect in the concentrations employed, nor did preincubation with reserpine block the serotonin effect. Acetylcholine, pitressin, posterior pituitary powder and extracts of posterior pituitary and hypothalamus did not enhance glucose oxidation by the anterior pituitary.

MANY studies have suggested that neurohumoral agents, perhaps carried in the hypothalamo-hypophysial portal circulation, influence the secretion of trophic hormones by the anterior pituitary gland (1). This hypothesis has been supported by experiments which indicate that the release of several pituitary hormones can be induced by systemic administration of neurohumoral agents. Thus, stimulation of adrenocorticotrophic hormone (ACTH) release by epinephrine (2) and serotonin (3) has been suggested by the observation that adrenal ascorbic acid depletion is produced by these substances in intact but not in hypophysectomized animals. Circulating thyroid stimulating hormone has also been found to be increased by epinephrine (4), as has luteinizing hormone secretion (5).

Because of the multiple systemic effects of epinephrine, serotonin and other neurohumoral agents which have been studied, it has been difficult to distinguish a direct action of these materials on the anterior pituitary from effects produced by secondary physiologic adjustments. The *in vitro* demonstration that a hypothalamic and neurohypophysial polypeptide can induce release of ACTH (6, 7) which can be potentiated by norepinephrine (6) has, thus, greatly strengthened the hypothesis of direct neurohumoral control of ACTH secretion. It has, in addition, emphasized

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the value of an *in vitro* approach to the problem of neurohumoral control of the pituitary.

Several studies with other endocrine glands have suggested another *in vitro* parameter. Thus, the demonstration that specific trophic hormones influence the *in vitro* glucose metabolism of thyroid (8) and adrenal (9) suggested that the glucose metabolism of the anterior pituitary might also be altered by specific regulatory agents. The possibility that neurohumoral agents, if important in regulating anterior pituitary function, might manifest or even mediate this effect by alterations in the glucose metabolism of this gland, prompted the following study.

EXPERIMENTAL PROCEDURES

Beef Pituitary

Beef pituitaries, removed within 15 minutes of the sacrifice of the animals by exsanguination, were transported on ice from the abattoir to the laboratory. Within two hours of the death of the animals the anterior pituitaries were trimmed, separated from the posterior and intermediate lobes, sliced with a Stadie-Riggs microtome, blotted, weighed, and placed in flasks for incubation.

Because of the variation in glucose oxidation in different slices from the same gland, and the known histological heterogeneity of the anterior pituitary gland, the following pooling procedure was generally employed. 1. Each gland was bisected along the median sagittal plane. 2. Sagittal slices were made. 3. Groups of 6 adjacent sagittal slices were segregated. 4. Each slice in the group of six was bisected. 5. The halves of the first slice were separated to begin one pair of piles and the halves of the second slice were separated to begin a second pair of piles. 6. The halves of the following four slices were then sequentially added to the piles so that each of the first two piles was ultimately derived from one half of slices 1, 3, and 5 and each of the second two piles was similarly derived from slices 2, 4, and 6. The glucose oxidation of the resulting four piles was remarkably similar when compared in the absence of stimulating agents.

In each portion of an experiment one member of each of the two pairs of piles was used for a control flask, and the other member of each pair for an experimental flask so that duplicate experimental observations could be compared with duplicate control observations on similar tissue. For each control flask there was an experimental flask containing a virtually identical aliquot of tissue. The weights of the piles in a group were similar and generally 50 mg./flask was used. Different pooling procedures were occasionally employed and this is indicated under the appropriate tables.

Posterior pituitaries, from which the intermediate lobe was removed, were sliced along the horizontal plane and 4 slices were obtained which were bisected and separated into 4 piles, each weighing approximately 20 mg., by a similar procedure.

In general the glands employed were from non-lactating cows but occasional experiments with steer and bull pituitaries provided similar results.

Rat Tissues

Fed male Sprague-Dawley rats weighing 150–250 gm. were sacrificed by decapitation.

Pituitary—Whole rat pituitaries weighing approximately 5 mg. were employed.

Brain—A single slice comprising both superior hemicortices was removed, fragmented with forceps, and the fragments grouped into 4 portions of similar weight, two of which served as controls. The weight of each portion in the different experiments varied from 15–25 mg.

Testis—The tunica albuginea was removed and approximately 100 mg. aliquots of tubules were separated by teasing and added to each flask.

Liver—Slicing and pooling procedures similar to those for the anterior pituitary were utilized. 100–200 mg. of tissue was placed in each flask.

Preparation of posterior pituitary and hypothalamic extracts

Beef hypothalami and posterior pituitaries, removed within 15 minutes of the sacrifice of the animals, were kept on ice and homogenized within 3 hours of the death of the animals. The "hypothalamus" was regarded as that medial area of the brain limited anteriorly by the optic chiasm, posteriorly by the mamillary bodies and superiorly by the massa intermedia. A portion of the material lateral to the hypothalamic nuclei was trimmed off before homogenization.

1,500 mg. of "hypothalamus" and 1,500 mg. of posterior pituitary were each homogenized in 1 ml. chilled distilled H_2O. The homogenates were washed from the homogenizer with 4 ml. chilled distilled H_2O, spun for 10 minutes at 1,000 g. in a refrigerated centrifuge at 0° C and the supernatant was removed and frozen. 0.1 or 0.5 ml. of the supernatant solution representing approximately 30 or 150 mg. of tissue was used in the experiments described. All experiments were done within several days of the preparation of the extracts.

Reagents

Glucose-1-C^{14}, glucose-6-C^{14}, and pyruvate-2-C^{14} were obtained from Orlando Research, Inc. Glucose-1-C^{14} obtained from the National Bureau of Standards was also used. Pitressin was obtained from Parke, Davis, and Co. DCI (1(3,4 dichlorophenyl) 2 isopropyl amino ethanol HCl), an adrenergic blocking agent, was the gift of Dr. Julius Axelrod, National Institutes of Health. Acetone dried posterior pituitary powder was the gift of Dr. Robert Bates, National Institutes of Health. All other reagents were obtained from usual commercial sources. The creatinine sulfate complex of serotonin (5-OH tryptamine) and the bitartrate salts of L-epinephrine and L-norepinephrine were used throughout. All reagents were dissolved in Krebs Ringer bicarbonate buffer and the pH brought to 7.4 before use.

Incubation and Counting Procedures

All experiments were done in 25 ml. Erlenmeyer flasks equipped with rubber stoppers from which glass center wells were suspended.

The flasks were gassed with 95% O_2, 5% CO_2 and incubated in a Dubnoff metabolic shaker at 37° C. The duration of incubation was 30 minutes unless otherwise indicated in the tables. After incubation the reaction was stopped by addition of 0.1 ml. of 10 NH_2SO_4. One ml. of hyamine (*p*-(diisobutyl-cresoxyethoxyethyl)dimethylbenzylammonium hydroxide) was added to the center wells and the flasks were shaken for 60 minutes at room temperature to trap $C^{14}O_2$ in the hyamine. The hyamine was then transferred to counting vials using 0.4% diphenyloxazole in toluene to rinse the center wells. Additional diphenyloxazole in toluene was added to the counting vial to make up a final volume of 15 ml. The vials were counted in a Packard liquid scintillation counter and results expressed as counts per minute in $C^{14}O_2$ generated per gm. (wet weight) of tissue during the total incubation period. The glucose concentration in all radioactive glucose experiments was 75 mg.% and the volume of medium was 2 ml. One-half microcurie glucose-1-C^{14} (approximately 5×10^5 c.p.m.) and 1.0 microcurie glucose-6-C^{14} (approximately 10^6 c.p.m.) were added to the appropriate flasks unless otherwise indicated in the tables. In experiments with radioactive pyruvate 0.5 microcurie pyruvate-2-C^{14} was used and the pyruvate concentration was 0.8 mg.%.

When glucose uptake was measured the glucose concentration in the flasks was 100

TABLE 1. EFFECT OF EPINEPHRINE, NOREPINEPHRINE AND SEROTONIN ON GLUCOSE AND PYRUVATE OXIDATION BY BEEF ANTERIOR PITUITARY

Label	Reagent	$C^{14}O_2$ (cpm/gm.)		Exper/Cont ×100	Mean*
		Control	Experimental		
Glucose-1-C^{14}	Epinephrine	6,276	14,407	230	
	Norepinephrine	3,718	8,320	224	
	Serotonin	5,393	15,777	293	
	Na Bitartrate	5,119	5,220	102	
	Creatinine + Na_2SO_4	3,848	3,737	97	
Glucose-6-C^{14}	Epinephrine	514	548	107	
		807	766	95	110
		1,812	2,294	127	
	Serotonin	636	547	86	
		1,138	1,458	128	104
		657	660	98	
Pyruvate-2-C^{14}	Epinephrine	32,780	36,221	110	94
		89,153	69,686	78	
	Serotonin	39,148	41,913	107	92
		98,175	74,550	76	

* Mean of Exp/Cont ×100.

All data are the average of closely agreeing paired duplicates. The concentration of all reagents was 2×10^{-4}M. 5×10^{5} cpm were added to the glucose-1-C^{14} flasks. 10^{6} cpm were added to the glucose-6-C^{14} flasks but the results were divided by 2 to make the specific activity comparable to that in the glucose-1-C^{14} experiments. No glucose was added to the pyruvate flasks. The variability in glucose oxidation by the controls for the different reagents in this and subsequent tables is due to both variation within and among glands.

mg.% and incubations were for 3 hours. One ml. aliquots of the incubation media were deproteinized by the method of Somogyi and glucose determined by the method of Somogyi and Nelson (10, 11) on duplicate aliquots of filtrate. In each experiment glucose determinations were made on the medium of flasks which had glucose with or without other reagents but to which no tissue was added. It was thus shown that 10^{-4}M epinephrine and serotonin did not interfere with the reducing substance method employed.

RESULTS

Epinephrine, norepinephrine and serotonin stimulate the oxidation of glucose-1-C^{14} to $C^{14}O_2$ by beef anterior pituitary (Table 1). This effect is

TABLE 2. EFFECT OF VARIOUS CONCENTRATIONS OF EPINEPHRINE AND SEROTONIN ON GLUCOSE-1-C^{14} OXIDATION BY BEEF ANTERIOR PITUITARY

Reagent	Concentration	$C^{14}O_2$ (cpm/gm.)		Exper./Cont. ×100
		Control	Experimental	
Epinephrine	2.5×10^{-5}	2,632	5,021	191
	8×10^{-6}	3,904	5,123	131
	2×10^{-6}	4,391	5,852	133
	2×10^{-6}	2,647	3,642	138
	5×10^{-7}	6,730	6,631	99
Serotonin	5×10^{-5}	5,177	10,702	207
	2.5×10^{-5}	3,555	5,958	168
	2×10^{-6}	4,940	5,418	110

All data are the average of closely agreeing paried duplicates.

TABLE 3. THE EFFECT OF EPINEPHRINE AND SEROTONIN ON BEEF ANTERIOR PITUITARY GLUCOSE OXIDATION DURING DIFFERENT INCUBATION PERIODS

Duration of incubation (Min.)	Reagent	Postion of label	$C^{14}O_2$ (cpm/gm.)		Exper./Cont. ×100
			Control	Experimental	
15	Serotonin	Glucose-1-C^{14}	1,900	5,835	307
30			4,791	13,983	292
60			16,612	31,075	187
120			34,082	59,277	174
180			16,371	29,784	182
90	Serotonin	Glucose-6-C^{14}	5,582	4,484	80
15	Epinephrine	Glucose-1-C^{14}	1,298	3,185	245
45			6,276	14,407	230
90			15,527	27,128	175
180			44,443	69,220	156
90	Epinephrine	Glucose-6-C^{14}	4,697	3,826	81

Control and experimental data are the average of closely agreeing paired duplicates. Epinephrine concentration was 2×10^{-4}M and serotonin concentration was 1×10^{-4}M. 0.5 μc. glucose-1-C^{14} or glucose-6-C^{14} was present in each flask.

demonstrable using 2×10^{-6}M epinephrine and 2.5×10^{-5}M serotonin (Table 2) and is apparent at 15 minutes (Table 3) which was the briefest duration of incubation employed. The effect persists with more prolonged incubations (Table 3). The oxidation of glucose-1-C^{14} is also stimulated in rat cerebral cortex (Table 4) and pituitary (Table 5) and, less prominently, in beef posterior pituitary (Table 5). $C^{14}O_2$ production from glucose-6-C^{14} by beef anterior pituitary (Table 1) and by rat cerebral cortex (Table 4)

TABLE 4. INFLUENCE OF EPINEPHRINE, NOREPINEPHRINE AND SEROTONIN ON GLUCOSE OXIDATION BY RAT CEREBRAL CORTICAL FRAGMENTS

Label	Reagent	Concentration	$C^{14}O_2$ (cpm/gm.)		Exper./ Cont. ×100
			Control	Exper.	
Glucose-1-C^{14}	Epinephrine	2×10^{-4}M	82,991	113,502	137
		2×10^{-5}M	34,217	70,702	207
		2×10^{-6}M	48,319	76,279	158
	Serotonin	2×10^{-4}M	67,980	130,408	192
		2×10^{-5}M	26,170	31,749	121
		2×10^{-6}M	63,300	66,200	105
	Norepinephrine	1×10^{-4}M	62,304	89,586	144
Glucose-6-C^{14}	Epinephrine	2×10^{-4}M	74,458 (6)	77,558 (6)	104
	Serotonin	2×10^{-4}M	65,065 (4)	66,261 (4)	102

The data represent the average of paired duplicate observations except where parenthesized numbers, indicating a greater number of determinations, are appended. In this latter case groups of paired duplicate experimental and control observations were made and the average of all of these is recorded. The paired duplicate experimental and control observations were made on fragments from the same cerebral cortex. Since the studies with different concentrations of reagents and with differently labeled glucose were made on different tissues, a precise quantitative comparison between these separate experiments cannot be made. 5×10^5 cpm glucose-1-C^{14} or glucose-6-C^{14} was present in each flask.

TABLE 5. STIMULATION OF GLUCOSE-1-C^{14} OXIDATION IN RAT PITUITARY AND BEEF POSTERIOR PITUITARY

Tissue	Reagent	Concentration	$C^{14}O_2$ (cmp/gm.)		Exper./Cont. ×100
			Control	Experimental	
Rat Pit.*	Serotonin	2×10^{-4}M	409,680	655,023	160
Beef Post. Pit.	Epinephrine	2×10^{-4}M	23,310	31,654	136
	Norepinephrine		17,456	21,042	121
	Serotonin		36,453	61,965	170

* Rat pituitaries were incubated for 45 minutes in the center wells of the flasks using 0.2 ml. of medium with 5×10^5 cpm glucose-1-C^{14} and a total glucose concentration of 71 mg.%. Results are the average of 3 control and 3 experimental observations. The usual procedure was followed with the posterior pituitary slices.

was not stimulated in 30 minute incubations with 2×10^{-4}M epinephrine or serotonin. Glucose-6-C^{14} oxidation by anterior pituitary was also not stimulated in 90 minute incubations (Table 3). Pyruvate-2-C^{14} oxidation by beef anterior pituitary was likewise not stimulated by 2×10^{-4}M epinephrine or serotonin in 30 minute incubations (Table 1). Sodium bitartrate and creatinine plus Na_2SO_4 were without effect (Table 1). No significant effect of epinephrine or serotonin on glucose uptake by beef anterior pituitary slices was demonstrated in three hour incubations (Table 6).

Epinephrine, norepinephrine and serotonin did not influence glucose-1-C^{14} oxidation by rat testis while, in rat liver, the catecholamines caused slight inhibition of $C^{14}O_2$ production (Table 7).

Acetylcholine, pitressin and posterior pituitary powder did not influence glucose-1-C^{14} oxidation by anterior pituitary and the supernatant from homogenates of hypothalamus and posterior pituitary caused slight inhibition (Table 8).

Several studies were done on the influence of blocking agents on the observed stimulation of anterior pituitary slices. No inhibition of the stimulation of glucose-1-C^{14} oxidation by 5×10^{-5}M epinephrine was found using 3×10^{-4}M dibenzyline or 4×10^{-4}M DCI. Five$\times10^{-5}$M ergotamine by itself inhibited glucose-1-C^{14} oxidation slightly but did not inhibit the

TABLE 6. LACK OF DEMONSTRABLE EFFECT OF EPINEPHRINE AND SEROTONIN ON GLUCOSE UPTAKE BY ANTERIOR PITUITARY SLICES

Reagent	Control mg. uptake/gm.	Experimental mg. uptake/gm.	Mean difference ±S.E.
Serotonin 10^{-4}M	2.53	2.91	0.39±0.38
Epinephrine 10^{-4}M	2.76	2.92	0.17±0.25

Tissue was prepared by bisecting all the slices from half of a gland and using half of these for a control and half for an experimental flask. 150–200 mg. of tissue was used per flask. 100 mg.% was present in the medium. Incubation duration was 3 hours. Each value is the mean of duplicate determinations done on 6 flasks. The fact that the experimental and control observations were paired permitted calculation of the difference between each paired experimental and control observation and then computation of the mean of these differences and of the standard error of this mean.

TABLE 7. $C^{14}O_2$ PRODUCTION FROM GLUCOSE-1-C^{14} BY RAT TESTIS AND LIVER

Tissue	Reagent	$C^{14}O_2$ (cpm/gm.)		Exper./Cont. ×100
		Control	Experimental	
Testis	Epinephrine	89,256*	95,240*	107
	Serotonin		89,257*	100
	Norepinephrine	81,540	77,081	97
Liver	Epinephrine	119,434	101,303	85
	Norepinephrine	121,533	101,091	83
	Serotonin	107,027	101,426	95

All data are the average of closely agreeing duplicates except for (*) where 7 samples of tubules were obtained from 1 animal, 3 of which were used as controls, 2 for epinephrine, and 2 for serotonin. The concentration of all reagents was 2×10^{-4}M. Testis was incubated for 30 minutes and liver for 90 minutes.

TABLE 8. GLUCOSE-1-C^{14} OXIDATION BY BEEF ANTERIOR PITUITARY IN THE PRESENCE OF OTHER AGENTS

Reagent	Concentration	$C^{14}O_2$ (cpm/gm.)		Exper./Cont. ×100
		Control	Experimental	
Pitressin	0.2 u./ml.	5,423	5,536	102
Posterior Pituitary Powder	1.0 mg./ml.	11,564	11,327	98
Posterior Pituitary Extract	0.1 ml./2 ml.	11,321	8,371	74
Hypothalamic Extract	0.1 ml./2 ml.	10,682	8,994	84
Acetylcholine	10^{-3}M	11,227	11,721	104

The acetylcholine was done in triplicate and is representative of a large number of experiments showing no effect with concentrations varying from 10^{-2} to 10^{-5}M. All other data are the average of closely agreeing paired duplicates. Incubation with posterior pituitary powder was for 120 minutes, whereas in the other experiments it was for 60 minutes.

stimulation produced by 2×10^{-4}M epinephrine. Preincubation for 12 minutes with reserpine phosphate (10^{-4}M) did not influence the stimulation induced by 10^{-4}M serotonin.

Whereas duplicate control and experimental observations agreed very closely in all the experiments reported, variability in glucose oxidation by *different* sets of controls, reflecting variation within and among glands, was not avoided by the pooling procedure employed.

DISCUSSION

The stimulation of glucose-1-C^{14} oxidation by anterior pituitary slices which was induced by epinephrine, norepinephrine and serotonin represents a possible biochemical correlate for the proposed physiological influence of these agents on this gland. One might, however, appropriately question the physiological significance of this finding since the concentrations of these materials required to produce an effect on anterior pituitary glucose oxidation are greater than those normally found in circulating blood. Thus, the normal plasma concentration of epinephrine and norepinephrine is in the range of 10^{-9}M (12) which is far less than the minimal concentration required to influence our system; and whole blood serotonin, although

normally present in 10^{-6}M concentrations, is virtually completely platelet bound (13). This objection may not, however, be valid, since the anterior pituitary may be exposed to larger concentrations of these agents than might be anticipated from systemic blood levels. Thus hypothalamus has been shown to contain more than 1 μg of norepinephrine (14) and serotonin (15) per gram of tissue (in the range of 10^{-6}M) and this concentration, which is greater than that found in other areas of the brain, suggests that the hypothalmo-hypophysial portal blood perfusing the pituitary may be unusually rich in these materials. Furthermore, the finding (16) that the pituitary rapidly concentrates an acute intravenous dose of epinephrine more intensely than any other organ studied, and to a level ten times as great as that found in the blood, implies that a systemic discharge of epinephrine may expose the pituitary to far more of this agent than is suggested by systemic plasma concentrations. Finally, the fact that the pituitary is a very heterogeneous tissue should also be considered when evaluating the concentrations of an agent required to produce an effect on this tissue as a whole. Thus it is possible that certain cell types of the pituitary may respond to smaller concentrations of these agents than those found necessary to produce an obvious effect in a slice which has many distinct and specialized components.

The lack of effect of these agents on testicular and hepatic glucose-1-C^{14} oxidation indicates that the phenomenon described is somewhat tissue specific although in liver the degree of dilution of labeled glucose by catecholamine stimulated glycogenolysis (which could lower the specific activity of the CO_2 generated) was not assessed. It is noteworthy, in this regard, that glycogen could not be demonstrated in testis, anterior pituitary or posterior pituitary.[1]

The demonstration of stimulation of glucose-1-C^{14} oxidation in brain may represent a biochemical correlate of the many physiological effects which these agents have on this organ (18). It is, furthermore, possible that the hypothalamus itself may respond to these agents in the same manner as cerebral cortex and anterior pituitary and that important endocrine effects may then also result in this more indirect way.

Brain and pituitary are not, however, the only tissue in which glucose-1-C^{14} oxidation is stimulated by these materials. Thus, it has been shown (19) that epinephrine, norepinephrine and serotonin produce a similar effect in dog thyroid. Since the minimum effective dose in thyroid is 10^{-4}M for each of these agents (19) the greater sensitivity of brain and anterior pituitary maintain for them a degree of exclusiveness. $C^{14}O_2$ production from both C-1 and C-6 labeled glucose has, furthermore, been stimulated in adipose tissue (20) by 10^{-6}M epinephrine. Since glucose-6-C^{14} oxidation was pre-

[1] Glycogen determinations were kindly performed by Dr. Hibbard Williams by the Anthrone Method (17).

dominantly stimulated in adipose tissue, whereas no stimulation of glucose-6-C^{14} could be demonstrated in pituitary or brain, the mechanism of action of epinephrine in these tissues may differ.

The mechanism of stimulation of glucose oxidation by the catecholamines and serotonin has not been completely elucidated by these studies. The finding that $C^{14}O_2$ production from glucose-1-C^{14} but not from glucose-6-C^{14} is stimulated by these agents suggests that the hexosemonophosphate pathway is being activated, but does not preclude an effect on other pathways as well (21). Since an appreciable stimulation of glucose-1-C^{14} oxidation by epinephrine and serotonin was readily demonstrable with three hour incubations whereas no similar increase in glucose uptake could be demonstrated, one must conclude that all pathways of glucose metabolism are not influenced to the same extent as those which express themselves by $C^{14}O_2$ production from glucose-1-C^{14}. The finding that pyruvate-2-C^{14} oxidation, measured in the absence of glucose in the medium, is not stimulated, suggests as does the glucose-6-C^{14} data, that the tricarboxylic acid cycle is not stimulated by these agents, although the possibility of dilution of the labeled pyruvate by increased generation of unlabeled pyruvate from endogenous substrates has not been excluded.

Whereas the established existence of a hexosemonophosphate pathway in anterior pituitary (22, 23) permits speculation concerning its stimulation by these neurohumoral agents, brain has been said to metabolize glucose almost exclusively via glycolysis and the tricarboxylic acid cycle (24, 25). The demonstration (26) that Synkavite can stimulate glucose-1-C^{14} but not glucose-6-C^{14} oxidation in brain has been interpreted to indicate that an alternate route of glucose oxidation not appreciably utilized under basal conditions may be rendered demonstrable by certain reagents. Our finding that glucose-1-C^{14} but not glucose-6-C^{14} oxidation is stimulated in brain by catecholamines and serotonin, may also be interpreted in this manner. This finding is particularly significant since the reagents which we have employed are of considerable physiological significance.

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THE SELECTIVE TESTICULAR EFFECTS OF CERTAIN BIS-(DICHLOROACETYL) DIAMINES

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ABSTRACT

N,N′-4-Xylylene-bis(N-ethyldichloroacetamide), Compound I, and N,N′-bis (dichloroacetyl)-1,8-octamethylenediamine, Compound II, produced in rats marked testicular weight regression associated with complete arrest of spermatogenesis. Compound II was approximately eight times more active, per mg., than Compound I. At maximal effects the seminiferous tubules consisted merely of a ring of Sertoli cells with spermatogonia interspersed. Leydig cell morphology and androgen production were not altered by antispermatogenic doses of either agent.

The weight of the adrenal glands was lowered by high doses of both compounds although the adrenals appeared normal histologically. The plasma corticosterone level of chronically medicated rats was not different from that of control animals. Also, the rise in plasma corticosterone concentration observed two hours after ACTH administration was not altered by prior medication with Compound II.

Concurrent injections of 10 R.U. per rat per day of commercial pituitary gonadotrophin (predominantly FSH) tended to enhance rather than reverse the testicular effects of Compound I. Also, testosterone propionate at 25 mg./kg./day increased the antispermatogenic action of Compound II, although the androgen by itself did not alter normal spermatogenesis.

The antispermatogenic activity of Compound I was not blocked by α-tocopherol, corn oil, ascorbic acid, calcium pantothenate, methionine, cysteine, ergothionine, lysine, arginine, histidine or spermine tetrahydrochloride. Neither compound blocked the growth of microorganisms when folic acid, calcium pantothenate, vitamin B_{12}, cystine or phenylalanine were limiting nutrilites.

Various screening tests indicated that Compounds I and II are not myotrophic, androgenic, estrogenic, progestational, nor glycogenic.

CERTAIN members of a novel series of bis-(dichloroacetyl) diamines synthesized (1, 2) in our laboratories have been observed (3) to curtail spermatogenesis in rats, rabbits, dogs and monkeys without producing discernible effects on other than testicular tissue. Extensive toxicological studies indicated that these compounds exerted a degree of selective effect on sperm producing cells not attained heretofore with agents such as ethionine (4) nitrofurans (5) and various radiomimetics (6, 7, 8). These initial observations prompted studies to determine the extent to which

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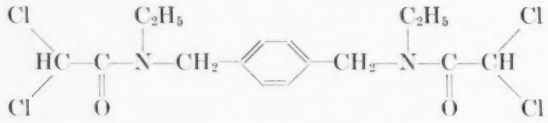
endocrine factors might be involved in the mediation of the antispermatogenic action of representative bis-(dichloroacetyl) diamines. Two types of compounds are included in this report.

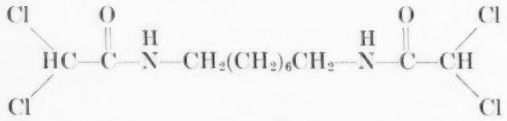
The formula for each is shown below:

Compound I



N,N′-bis(dichloroacetyl)-N,N′-diethyl-1,4-xylylenediamine

Compound II



N,N′-bis(dichloroacetyl)-1,8-octamethylenediamine

Compound I is a very potent chemotherapeutic agent against amebae in animals and man, whereas Compound II is only very weakly active, if active at all, as an amebicidal agent (9).

Basic questions towards which the experiments included in this report were directed are: Is the hormone producing capacity of the testes affected by these agents? Can the antispermatogenic action of these compounds be explained on the basis of altered gonadotrophic function of the pituitary or on the basis of any possible hormonal activity? Is the effect on sperm producing cells reversed or prevented by concurrent administration of essential metabolites?

The effect of these bis-(dichloroacetyl) diamines on fertility of laboratory animals, on the course of pregnancy and on embryonic development will be the subject of a subsequent report.

MATERIALS AND METHODS

Male and female rats of the Sprague-Dawley strain were housed in temperature-regulated and air-conditioned quarters. Laboratory chow and water were provided ad libitum.

Effects on testicular function and morphology. Male rats (170–190 gms.) were used in this study. The test agents were prepared as suspensions in water containing 1% gum tragacanth. Compound I was administered orally to groups of male rats at dose levels of 125, 250, 500 and 1000 mg./kg. and Compound II at 62.5, 125 and 250 mg./kg. daily except Sunday for 3 weeks. The rats were autopsied approximately 24 hours after the last medication and the testes, ventral prostate, seminal vesicles, adrenals, thymus and pituitary were removed, cleaned and weighed on a micro-torsion balance. At autopsy, the testes, adrenals and thyroid glands were fixed in Zenker-formalin, sectioned and stained with hemotoxylin-eosin. Initial and final body weights were recorded. The anti-

spermatogenic effects of these test agents were assessed on the bases of decreased testicular weights as well as on histological evaluation of degree of spermatogenic arrest.

Effect of Compounds I and II on adrenal cortical function. The plasma corticosterone level of rats was determined following various periods of medication with Compound I or II. The method of analysis of corticosterone was that described by Silber *et al.* (15). The responsiveness of the adrenal cortex to ACTH ascertained by elevated plasma corticosterone levels was determined in male rats medicated with the antispermatogenic compounds daily for 3 days prior to ACTH challenge.

Pituitary gonadotrophin content. The pituitary glands from various experimental groups were placed in 0.9% saline and homogenized in McShan-Erway homogenizers. The homogenates were quick-frozen on dry ice and stored in a freezer until ready for assay. The gonadotrophin content of the homogenates was determined in young hypophysectomized female rats (50–55 gms.). The equivalent of 0.83 pituitary glands was injected subcutaneously as 6 spaced injections over a 72-hour period. The rats were autopsied approximately 48 hours after the last injection and the ovaries removed, cleaned and weighed on a micro-torsion balance to the nearest 0.1 mg. The results have been expressed as units of gonadotrophin. A unit is defined as that amount of gonadotrophin which when injected according to the above described procedure produces a 100% increase in ovarian weight over that observed for saline injected control hypophysectomized rats.

Effect of pituitary gonadotrophin on antispermatogenic action of Compound I. Immature male rats (75–85 gm.) were used in this experiment. Compound I was administered orally by stomach tube to 3 groups of 7 rats each at dose levels of 125, 250 and 500 mg./kg. daily except Sunday for 3 weeks. The drug was given as a fine suspension in 1% gum tragacanth. Three other groups of 7 rats each, which also received Compound I at dose levels of 125, 250 and 500 mg./kg., were given in addition 10 R.U. per rat of pituitary gonadotrophin (Squibb, predominantly FSH) as a subcutaneous injection daily except Sunday for 3 weeks. Another group of 7 rats received the pituitary gonadotrophin alone. Seven other rats which received the vehicle only served as controls. The rats were autopsied on the 22nd experimental day, approximately 24 hours after the last medication. The testes and ventral prostate were removed, cleaned and weighed on a micro-torsion balance. The testes were fixed in Zenker-formalin, sectioned and stained with hematoxylin-eosin.

The combined effects of Compound II and testosterone propionate on testes of young male rats. Young male rats weighing 100–125 gm. were divided into four groups of 10 rats each. One group was used for control and received diluents only. Compound II, 125 mg./kg./day, was administered orally to two groups, one of which in addition was injected subcutaneously with testosterone propionate at a dose of 25 mg./kg./day. A fourth group received testosterone propionate alone. Medications were administered concurrently six days a week for 3 weeks. At autopsy selected organs were weighed and fixed as described above.

Attempts to prevent the antispermatogenic effects of Compound I. Attempts were made to prevent the antispermatogenic effects produced by the oral administration of Compound I to adult male rats (170–190 gm.) at a dose level of 500 mg./kg. daily except Sunday for 3 weeks. Dietary supplements used and the percentage levels in the diet were as follows: calcium pantothenic acid, 0.04; ascorbic acid, 0.03 and 0.13; methionine, 0.33 and 1.3; cysteine, 1.0; lysine, 1.0; arginine, 1.0; and histadine, 1.0. Also, α-tocopherol acetate (200 mg./kg./day); ergothionine (10 mg./kg./day), spermine tetrahydrochloride (10 mg./kg./day) and corn oil (1 ml./rat/day) were administered subcutaneously or orally concurrent with Compound I. The rats were autopsied approximately 24 hours after the last medication and the testes removed, cleaned and weighed. The testes were fixed in Zenker-formalin, sectioned and stained with hemotoxylin-eosin. Testicular

weights and histological evaluation of representative sections served as the basis for assessment of prevention of antispermatogenic effects.

Assessment of hormonal effects. Increases in the weight of the levator ani muscle and ventral prostate glands of immature castrated male rats were used as indices for assessment of myotrophic and androgenic activities, respectively (10). Estrogenic activity was determined on the basis of the cornification response of the vaginal epithelium of castrated female rats (11). Stimulation of uterine growth of immature female rats (40–47 gm.) was used to assess uterotrophic activity (12). Proliferation of the endometrium of the estrogen primed immature rabbit uterus as described by McPhail (13) was used for assessment of progestational activity. Compounds I and II were studied for their ability to deposit liver glycogen in adrenalectomized rats according to the method of Olson *et al.* (14).

RESULTS

1) *Effects on testicular morphology and function.* Daily oral medication of young mature male rats for three weeks with Compound I or Compound II produced marked testicular weight regression (cf. Table 1). Compound II was approximately eight times more potent than Compound I in this respect. Grossly, the testes from treated rats appeared hyperemic and were quite flaccid when compressed. Histologically, the spermatogenic cells exhibited degenerative changes which in degree were related to size of dose. Proceeding from low to high doses the following tubular changes were apparent: 1) disappearance of spermatids and "cap cells," 2) reduction in number of secondary spermatocyte cells, 3) appearance of giant cells among a sparse population of primary spermatocytes, and finally 4) a ring of tubular basal cells composed of Sertoli cells interspersed with spermatogonia. As these stages of spermatogenic arrest progressed, the dimensions of the seminiferous tubules decreased so that at full effect the cross-sectional area was generally $\frac{1}{3}$ to $\frac{1}{2}$ that of tubules from control rats. The interstitial (Leydig) cells at all dose levels of either compound appeared normal in appearance. However, the number of interstitial cells per microscopic field was noticeably greater in testes from rats treated with 125 mg./kg. of Compound II or 1000 mg./kg. of Compound I compared with testicular sections from normal rats. The observed difference upon preliminary estimation would appear to be greater than could be accounted for on the basis of a concentration effect due to the marked reduction in size of the testes. Many of the above described testicular changes can be seen in Figures 1 through 8.

Evidence that the hormone secreting capacity of the testes was not disturbed by medication with bis-(dichloroacetyl) diamines was obtained from analysis of the sex accessory organ weights presented in Table 1. The data show that the weights of the ventral prostate and seminal vesicles were not significantly different from respective control weights ($P > .05$). It should be noted, however, that the ventral prostate glands showed a tendency to become smaller with increasing doses, particularly with Compound I. This trend may be a reflection of the slight suppression of body

TABLE 1. TESTICULAR AND OTHER ORGAN WEIGHTS OF RATS ADMINISTERED BIS-(DICHLOROACETYL) DIAMINES

Compound	Dose, mg./kg./day	Total number of rats	Body weight (gm.)		Organ weights (Mean ±S.E.)					
			Initial	Final	Testes (gm.)	V. prostate (mg.)	S. vesicles (mg.)	Adrenal (mg.)	Thymus (mg.)	Pituitary (mg.)
Control	0	32 (4)[1]	190	302	3.04 ± .14[2]	235 ± 20	243 ± 16	42.4 ± 2.5	512 ± 48	10.9 ± .48
Compound I	125	12 (2)	197	304	2.99 ± .11	225 ± 12	219 ± 9.9	42.3 ± 2.1	540 ± 63	11.8 ± .84
	250	22 (3)	195	288	2.56 ± .15†	209 ± 12	221 ± 10	35.4 ± 1.7†	518 ± 31	11.7 ± .54
	500	21 (3)	192	290	2.47 ± .18†	202 ± 11	231 ± 12	37.6 ± 1.4	570 ± 35	11.0 ± .68
	1000	16 (2)	190	277	2.02 ± .20‡	198 ± 15	237 ± 17	36.2 ± 1.3†	542 ± 35	10.6 ± .42
Control	0	24 (3)	202	311	3.15 ± .11	238 ± 15	237 ± 16	47.4 ± 3.3	503 ± 60	11.8 ± .44
Compound II	62.5	30 (4)	199	311	2.32 ± .16‡	224 ± 19	260 ± 13	41.1 ± 2.2	538 ± 39	10.1 ± .48
	125	30 (4)	199	302	2.06 ± .16‡	216 ± 18	254 ± 16	41.1 ± 2.1	535 ± 47	10.4 ± .45
	250	30 (4)	198	291	1.88 ± .11‡	218 ± 10	266 ± 14	38.4 ± 2.0†	516 ± 41	10.0 ± .53

† Significantly different from control value with P value of $< .05$ but $> .01$.
‡ Significantly different from control value with P value of $< .001$.
[1] The number in parenthesis indicates the number of experimental groups from which the data were derived.
[2] These values are the averages of the means and standard errors of all corresponding experimental groups.

TABLE 2. PLASMA CORTICOSTERONE LEVEL OF MALE RATS TREATED WITH BIS-(DICHLOROACETYL) DIAMINES

Compound	Dose, mg./kg./day	Duration of test	Number of rats	Plasma corticosterone μg./100 ml. Mean ± S.E.
None	0	3 wks.	9	131 ± 11
Compound I	500	3 wks.	7	123 ± 10
None	0	12 wks.	5	158 ± 13
Compound II	62.5	12 wks.	4	166 ± 9.0
None	0	3 days	6	45 ± 3
ACTH[1]	4 units[1]	3 days	6	85 ± 3
ACTH + Compound II	4 units + 125	3 days	4	92 ± 4

[1] HP Acthar® Gel administered subcutaneously on the third day of the experiment.

weight gain recorded. If so, it is interesting to note that the seminal vesicles did not reflect this change.

2) *Effects on other endocrine organs.* The mean wet weights of the adrenals, thymus and pituitaries from the experimental animals are presented in Table 1. The adrenal glands of rats medicated with high doses of Compound I or II were noticeably smaller than the control glands. Histologically, the smaller glands exhibited no readily apparent difference from normal adrenals. Plasma corticosterone levels of male rats medicated daily except Sunday for 12 weeks with 62.5 mg./kg. of Compound II were not different from those of unmedicated control rats. Also, Compound I exerted no effect on plasma corticosterone levels of rats medicated for three weeks with 500 mg./kg./day. In acute experiments medication of male rats with Compound II three days prior to ACTH injections did not modify the adrenal response to the trophic hormone as evidenced by elevated plasma corticosterone concentrations. (These data are shown in Table 2.) The fact that the thymic weights of medicated animals (cf. Table 1) were not significantly different from control thymic weights lends support to the evidence that adrenal cortical function, at least as it relates to the production of glucocorticoids, is not altered by these bis-(dichloroacetyl) diamines.

The data in Table 1 indicate that the weight of the pituitary glands was not modified by the test drugs. Important in this connection are the observations that the gonadotrophin content of pituitaries from male rats medicated with Compound I or II was within normal limits as can be ascertained from the data in Table 3.

Examination of the thyroid glands in situ as well as histological studies revealed no discernible differences in appearance and cellular architecture of thyroid tissue from control and medicated rats.

3) *The effect of pituitary gonadotrophin on antispermatogenic action of bis-(dichloroacetyl) diamines.* Even though available evidence suggested that

TABLE 3. THE GONADOTROPHIN CONTENT OF PITUITARIES FROM MALE RATS ADMINISTERED BIS-(DICHLORACETYL) DIAMINES

Experimental group	Dose, mg./kg./day	Pituitary weight (mg.) Mean ±S.E.	Number of pituitaries per hypox. rat	Gonadotrophin content		
				Ovarian weight (mg.)	Units[1]/ pituitary	Units/ 100 mg. pituitary
Control	0 (9)[2]	10.4 ± .56	0.83	26.9 ±2.6[3]	3.00	28.8
Compound I	250 (10)	10.4 ± .58	0.83	25.7 ±2.6	2.82	27.1
	500 (10)	10.5 ± .55	0.83	31.0 ±2.4	3.65	34.8
	1000 (10)	11.5 ± .40	0.83	26.7 ±1.8	2.98	25.9
Control	0 (6)	11.0 ± .41	0.83	23.8 ±2.5[4]	3.61	32.8
Compound II	62.5 (6)	10.8 ±1.2	0.83	24.1 ±2.4	3.74	32.5
	125.0 (5)	11.4 ± .39	0.83	28.7 ±2.3	4.46	38.0

[1] A unit of gonadotrophin is defined as that amount of gonadotrophin which when injected subcutaneously as 6 spaced injections over a 72 hr. period produces a 100% increase over control in weight of ovaries of hypophysectomized rats weighing 50–60 gms.
[2] The number in parentheses indicates number of animals in each group.
[3] Ovarian weight of control hypophysectomized group: 7.7 ± .15.
[4] Ovarian weight of control hypophysectomized group: 5.9 ± .10.

pituitary gonadotrophic function was not altered by Compound I or II, the possibility remained that the action of gonadotrophin, particularly of FSH, on the seminiferous tubules might be blocked by these agents and that the blockade might be overcome partially, if not completely, by administering large doses of gonadotrophin. To test this possibility immature male rats were administered Compound I daily for three weeks with and without a commercial preparation of pituitary gonadotrophin containing predominantly FSH. The results of this experiment are presented in Table 4. Compound I at oral dose levels of 125, 250 and 500 mg./kg. administered daily except Sundays for 3 weeks brought about highly significant ($P < .01$) weight regressive changes of the testes without affecting the weight of the ventral prostate gland. The concurrent injection of pituitary gonadotrophin (FSH) at a dose level of 10 R.U./rat did not prevent the testicular weight reduction observed with the diamine. It should be noted that the mean testicular weight of the rats which received the combination of gonadotrophin and Compound I was less in each instance than that of the corresponding group of rats administered the drug alone. The mean differences, however, were not statistically significant.

The marked weight increase of the ventral prostate glands which accompanied the gonadotrophin regimen indicates that this particular preparation of gonadotrophin contained considerable amounts of interstitial cell stimulating hormone (ICSH). The observed prostate weight increase was not significantly altered by concurrent treatment with Compound I although a trend towards a weight reduction was noticeable.

The results of this experiment indicate that a physiologically active dose of pituitary gonadotrophin did not prevent the testicular effect of the

TABLE 4. THE COMBINED EFFECTS OF COMPOUND I AND PITUITARY GONADOTROPHIN ON TESTICULAR AND VENTRAL PROSTATE WEIGHTS OF YOUNG MALE RATS

Dose		No. of rats	Body weight (gms.)		Organ weights (Mean ±S.E.)	
Compound I, mg./kg./day, i.g.	Pituitary gonadotrophin (FSH), Units/rat/day, s.c.		Initial	Final	Testes (gm.)	V. prostate (mg.)
0	0	7	80	198	2.55 ± .06	74.2 ± 8.5
125	0	6	80	193	1.96 ± .10[1]	78.5 ± 5.4
250	0	5	79	195	1.87 ± .20	93.2 ± 11.0
500	0	5	80	196	1.59 ± .10	81.9 ± 20.0
0	10	4	80	207	2.47 ± .08	193.8 ± 20.8
125	10	7	79	190	1.78 ± .10[2]	159.0 ± 16.1
250	10	7	80	182	1.33 ± .10	153.8 ± 12.0
500	10	6	80	187	1.25 ± .07	141.1 ± 12.8

[1] The singly underlined values are statistically different from the respective non-medicated control values with P value of 0.02 or less.

[2] The doubly underlined values are statistically different from the respective FSH treated control values with P value of 0.02 or less.

antispermatogenic agent. Neither did Compound I interfere with the interstitial cell stimulating properties of the particular gonadotrophin preparation used.

The above described gross morphological changes were reflected in histological sections of the testes shown in Figures 9 through 16. The seminiferous tubules from testes of rats medicated with Compound I generally were smaller in diameter than normal and contained very few cells advanced beyond the primary spermatocyte stage of development (Figs. 11

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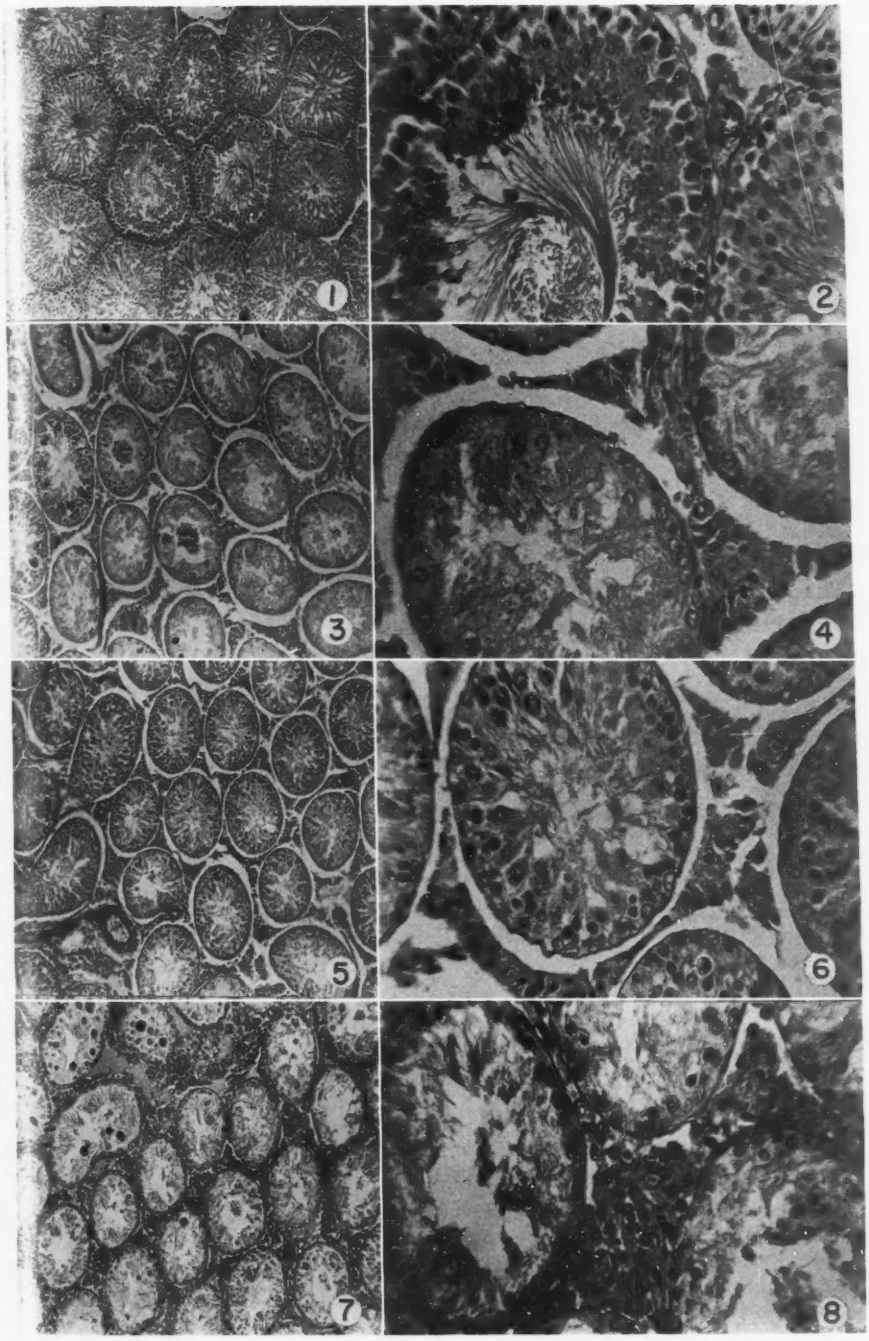
FIGS. 1 and 2. Testicular sections of control rat showing normal spermatogenesis and cellular architecture. H and E, ×100 and ×430.

FIGS. 3 and 4. Testicular sections of experimental rat after 21 days of oral medication with 1000 mg./kg./day of Compound I. Notice the decreased diameter of the seminiferous tubules, the sparce population of sperm producing cells, an occasional giant cell and an abundance of interstitial tissue. H and E, ×100 and ×430.

FIGS. 5 and 6. Testicular sections of experimental rat after 21 days of oral medication with 62.5 mg./kg./day of Compound II. The tubules are devoid of fully formed sperm and few cells have advanced beyond the primary spermatocyte stage. Interstitial cells appear normal. H and E, ×100 and ×430.

FIGS. 7 and 8. Sections of testis from a rat medicated orally for 21 days with Compound II at 125 mg./kg./day showing marked tubular degeneration, giant cells and abundant interstitial tissue. H and E, ×100 and ×430.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and 12). The concurrent administration of gonadotrophin appeared to enhance the testicular effects of the substituted diamine in that most of the tubules consisted merely of a thin ring of Sertoli cells and spermatogonia with an occasional giant cell. In addition, the tubules were smaller than those of rats medicated with Compound I alone. The apparent enhancement by pituitary gonadotrophin of the antispermatogenic effect of Compound I correlated with the testicular weight differences reported above.

4) *The combined effects of Compound II and testosterone propionate on testes of young male rats.* Compound II at 125 mg./kg./day, orally, over a 21-day period produced a marked decrease of testicular weight as indicated in Table 5. Histologically, the diameter of the seminiferous tubules was considerably reduced and the cell population within the tubules was composed mainly of spermatogonia and primary spermatocytes. The interstitial cells appeared normal. The weights of the sex accessory organs, thymus, adrenal and pituitary were not altered.

The testicular weight of testosterone treated rats was significantly less than that of control animals. The seminiferous tubules were smaller in cross section but spermatogenesis was fully maintained. The lumina of many tubules were filled with sperm creating the characteristic "swirl" of closely packed sperm tails. The interstitial cells were sparse and pyknotic. As expected, testosterone propionate at 25 mg./kg./day produced marked stimulation of the sex accessory organs and considerable reduction in thymus weight (cf. Table 5).

The testes of rats medicated concurrently with Compound II and testosterone propionate were greatly reduced in size and weight (Table 5). The seminiferous tubules were small in diameter and sparsely populated with sperm producing cells. In many tubules only an occasional spermatogonia could be seen among Sertoli cells giving the tubules the appearance of

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FIGS. 9 and 10. Sections of testis of control rat showing active spermatogensis and normal cellular architecture. H and E, ×100 and ×430.

FIGS. 11 and 12. Testicular sections of experimental rat medicated orally for 21 days with Compound I at 500 mg./kg./day. Spermatogenesis is arrested at the primary spermatocyte stage. Some tubules contain a few scattered secondary spermatocytes. The interstitial tissue appears normal. H and E, ×100 and ×430.

FIGS. 13 and 14. Testicular sections of experimental rat medicated for 21 days with a combination of Compound I at 500 mg./kg./day, orally, and pituitary gonadotrophin at 10 rat units/rat/day, subcutaneously. The small ring-like seminiferous tubules in most instances consist merely of a basement membrane with an overlying row of spermatogonia and Sertoli cells. The interstitial tissue is plentiful. H and E, ×100 and ×430.

FIGS. 15 and 16. Sections of testis of rat injected with pituitary gonadotrophin over a 21 day period at a dose level of 10 rat units/rat/day. Spermatogenesis is active through all stages. H and E, ×100 and ×430.

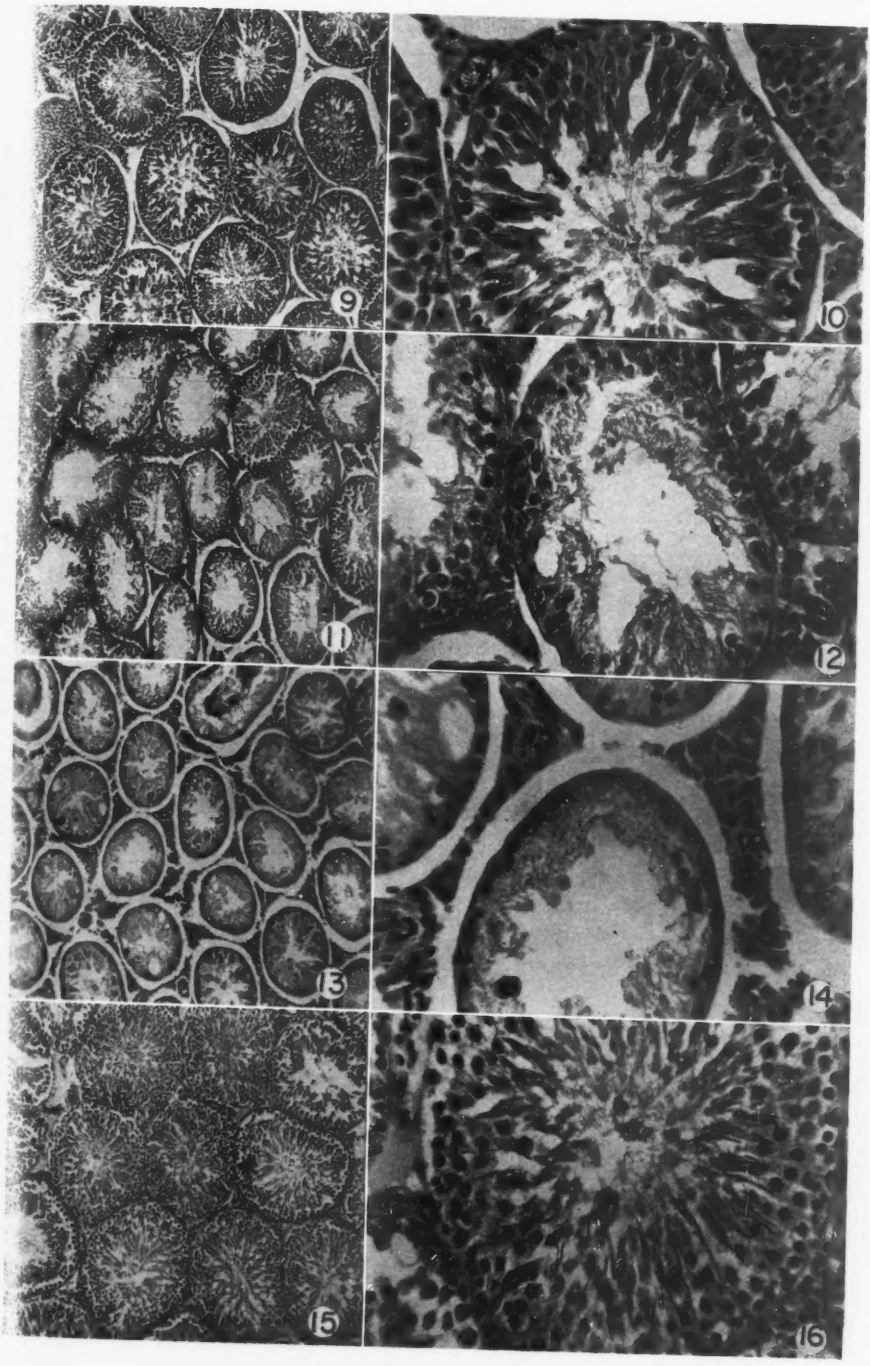
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TABLE 5. THE COMBINED EFFECTS OF COMPOUND II AND TESTOSTERONE PRIOPONATE ON TESTES AND OTHER ORGANS OF YOUNG MALE RATS

Compound	Dose, mg./kg./day	No. of rats	Body weight (gm.)		Organ weights (Mean ± S.E.)					
			Initial	Final	Testes (gm.)	V. prostate (mg.)	S. vesicles (mg.)	Thymus (mg.)	Adrenal (mg.)	Pituitary (mg.)
None	—	10	114	244	2.76 ± .09	141 ± 6.1	163 ± 9.9	511 ± 24	35.0 ± 1.8	8.0 ± .4
Compound II	125	10	114	239	1.42 ± .05	155 ± 17	176 ± 15	529 ± 27	33.6 ± 2.2	8.7 ± .6
Testosterone propionate	25	10	114	229	2.41 ± .04	368 ± 15	350 ± 8.7	184 ± 16	36.2 ± 1.6	8.0 ± .2
Compound II + Testosterone priopionate	125 25	10	114	204	1.15 ± .09	332 ± 18	393 ± 15	148 ± 15	34.3 ± 1.4	6.8 ± .3

thin ring-like structures. In other tubules marked degenerative changes were noticed evidenced by the coalescing of spermatocytes to form "giant cells" many of which contained from 10 to 20 nuclei. The number of interstitial cells per microscopic field was greater than that seen in the testosterone treated series. However, the appearance of the Leydig cells was similar to that observed following androgen treatment in that many of these cells were irregularly shaped and pyknotic. The testosterone induced changes in the sex accessory organs and thymus were not modified by concurrent oral administration of Compound II.

These results indicate that the antispermatogenic action of Compound II was increased by concurrent injection of testosterone propionate at a dose level of 25 mg./kg./day which dose by itself permitted normal spermatogenesis.

5) *Attempts to prevent the testicular effects of Compound I.* Concurrent administration of α-tocopherol, corn oil, ascorbic acid, calcium pantothenate, methionine, cysteine, ergothionine, spermine, lysine, arginine or histidine did not prevent the testicular weight regression produced in rats by Compound I. Histologically, the antispermatogenic effect of this diamine was not altered by any of the above agents.

6) *Antimetabolite studies with Compound I and II and Win 18,446.* Concentrations of 200 mcg. of Compound I or II per ml. of incubation medium did not inhibit the growth of S. faecalis (#8043), L. casei, L. leishmania (#7830), L. mesenteroides (P-60) or L. arabinosus (17-5) when folic acid, calcium pantothenate, B_{12}, cystine or phenylalanine, respectively, were employed as the limiting nutrilite.

7) *Tests for hormonal activities.* Neither of these antispermatogenic agents increased the size of the levator ani, ventral prostate or seminal vesicles of young castrated rats. The vaginal epithelium of castrated female rats remained atrophic during medication with Compound I or II. Neither compound was uterotrophic in immature rats; nor did the endometrium of estrogen-primed young rabbits exhibit progestational proliferation following medication with these agents. The glycogen content of livers of adrenalectomized rats was not altered during fasting. These observations indicate that Compounds I and II are not myotrophic, androgenic, estrogenic, progestational nor glycogenic.

DISCUSSION

The results of toxicological and allied studies reported earlier (3) with these bis-(dichloroacetyl) diamines indicated that sperm producing cells may be the only mammalian tissue elements vulnerable to chemical attack by these agents. The antispermatogenic dose levels ranged from 31 to 500 mg./kg. whereas the acute oral LD_{50} in mice was observed to be greater than 12,000 mg./kg. This marked difference in dose required for testicular

effects compared with the amount which can be tolerated by animals indicates the extent to which the sperm cells are selectively attacked. Unlike most other cells of the body, the sperm producing cells of the testes depend upon an adequate balance between gonadotrophic and testicular hormones for maintenance of a high level of proliferative activity (16, 17, 18, 19). The extent to which spermatogenesis is dependent upon these hormonal influences has not been clearly established. It is evident from the studies reported here that the administration of pituitary gonadotrophin (predominantly FSH) does not prevent the antispermatogenic effects of these agents. In fact, the gonadotrophin appeared to enhance the testicular effects of Compound I. This enhancement by gonadotrophin, if real, could be the result of 1) a direct effect whereby the gonadotrophin could speed up the rate of depletion of tubular components by increasing the proliferation of sperm producing cells which then become susceptible to the selective action of the diamine or 2) an indirect effect in some way related to alteration in pituitary or gonadal hormone production. The latter possibility is corroborated by the observation that testosterone propionate also enhanced the antispermatogenic effects of Compound II even though when given alone the androgen permitted the maintenance of normal spermatogenic activity. The ability of testosterone to enhance the antispermatogenic activity of bis-(dichloroacetyl) diamines may be due to an increased requirement by the sperm producing cells for one or more metabolites the utilization or synthesis of which is blocked by the antispermatogenic diamines.

Failure to reverse the antispermatogenic effects of bis-(dichloroacetyl) diamines by concurrent administration of various nutrients and metabolites selected somewhat arbitrarily suggests that the alleged limiting metabolite may be relatively specific for sperm producing cells. In this respect the site of action of the bis-(dichloroacetyl) diamines would appear to be different from that indicated for other substances known to have antispermatogenic activity. Ellman *et al.* (20) have reported that the antispermatogenic action of nitrofurans was prevented by supplementing the diet with cysteine. Methionine is known (21, 22) to prevent the antispermatogenic as well as other toxic effects of ethionine. Neither cysteine nor methionine prevented the testicular effects of bis-(dichloroacetyl) diamines. A variety of drugs are reported (6, 7, 8) to arrest spermatogenesis by exerting a radiomimetic effect on sperm producing cells. Notable among these is the compound triethylenemelamine (TEM). Apparently TEM exerts a damaging effect upon mature spermatozoa as well as upon spermatogonia whereas the compounds of the present series exert their primary effect upon spermatocytes. The results of studies to be reported in a subsequent paper indicate that sterility is induced in male rats only after sufficient time has elapsed for the compounds to disrupt spermatogenesis.

It is concluded that the bis-(dichloroacetyl) diamines constitute a class

of compounds with marked antispermatogenic activity divorced from significant effects upon other tissues of the body. It is anticipated that future investigations of the actions of these diamines will provide information which will contribute materially to the understanding and control of the biochemical and physiological aspects of spermatogenesis and male reproductive phenomena.

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TSH REBOUND PHENOMENON IN THE RAT ADENOHYPOPHYSIS[1]

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ABSTRACT

The dynamics of pituitary TSH secretion have been studied in the female rat throughout prolonged goitrogenesis (3 weeks to one year) and during readjustment of the pituitary-thyroid system after removal of the goitrogenic stimulus (2–21 days). TSH levels in sera of rats 5–15 months old are relatively constant but hormone concentration in the adenohypophysis is more variable and falls substantially in the old animal. With chronic PTU treatment, blood thyrotrophin levels increase 70–300% and pituitary hormonal stores decrease to less than 5% of normal. Goitrogen withdrawal results in heightened accumulation of hormone stores in the pituitary (4–8 fold increase) with peak of rebound at 6–12 days. TSH rebound in the adenohypophysis is associated with return of AF-positive glycoprotein granulation in the thyroidectomy cell. Blood TSH titers during pituitary repletion remain at nearly normal levels. Doses of triiodothyronine and thyroxine which normally induce almost complete disappearance of thyrotrophin from blood and hypophysis are relatively ineffective in preventing the TSH rebound phenomenon in goitrous rats. The rebound effect is not induced in normal rats 10 days after cessation of thyroid hormone treatment.

A "differential threshold" hypothesis of thyroid hormone function is proposed to explain the asynchronism in the dynamics of TSH secretion after goitrogen withdrawal. It is postulated that the momentum of TSH hypersecretion in the pituitary temporarily "overshoots" despite release from PTU and that the synthetic and storage phases are abnormally sensitive at this time to the low level of thyroid hormone produced by the unblocked gland. With subsequent rise in circulating levels of thyroid hormone, production and release of TSH are brought into balance and a steady state is re-established. Further increase in thyroid hormone to excessive levels inhibits both synthesis and release of thyrotrophin by the adenohypophysis.

CUMULATIVE efforts by many investigators have clearly established for the rodent the complex pattern of pituitary-thyroid interaction and the relationship of the hypothalamus to it (1–3). Inability to measure rates of thyrotrophin secretion by the adenohypophysis still poses a hindrance to unravelment of the mechanisms governing the interplay. Some aspects of this problem have received partial clarification from recent studies on the hypothalamo-hypophysial-thyroid apparatus of the rat. Experimental analysis of the neuroendocrine relationship by electrocautery

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and correlation of blood and pituitary TSH levels with thyroid activity permitted quantitative definition of the contribution made by neural and glandular components of the system (4–5). The hypothalamus was found to have a modulating influence on thyrotrophin secretion but apparently little or none on the pituitary-thyroid servomechanism itself. Lesions normally interfering with pituitary TSH function did not prevent massive discharge of the hormone with propylthiouracil treatment nor hinder its reaccumulation after goitrogen withdrawal. In the latter instance, TSH content of the adenohypophysis rebounded to unusually high levels, suggesting temporary asynchronism in thyrotrophin secretion.

The induction of pituitary TSH rebound in goitrous rats following removal of the goitrogenic stimulus represents an experimental approach through which the synthetic, storage and release phases of trophic hormone secretion might be profitably studied. The present investigation attempts to assess the significance of the rebound phenomenon and the dynamics of thyrotrophin secretion throughout prolonged goitrogenesis and during rehabilitation of the pituitary-thyroid system. The duration of the TSH effect and the sequential changes in hormone levels in the circulation will be determined simultaneously and correlated further with concurrent morphologic alterations in thyroid and adenohypophysis. The effects of normally suppressive doses of exogenous thyroid hormone on pituitary TSH function will also be tested in normal and in goitrous rats during the rebound phase. Some of the results have already been described (6).[2]

MATERIALS AND METHODS

Approximately 300 albino female rats (Wistar strain) ranging from 5–15 months of age (175–450. gm body weight) were used in the experiments. Groups A–H served as normal weight and age controls to experimental groups I–M which were fed propylthiouracil (PTU; .1%) in the ration of Purina Dog Checkers for periods of 18–130 days. The basic diet and tap water were always available in unrestricted amounts. Changes in pituitary and serum TSH levels after goitrogen withdrawal were studied in 5 other groups (N–R) of 10 rats each (30–50 days on PTU) by returning them to the normal diet for periods of 3–20 days before sacrifice. The effects of exogenous thyroid hormones on TSH rebound in the adenohypophysis were tested in two long term experiments. In the first, rats which had been on PTU for 90 days were returned to the normal diet and were administered daily 10 μg of sodium L-triiodothyronine (T_3) in physiological saline (pH 8.5) for 10 days to the end of the experiment by subcutaneous injection. A goitrous control group similarly released from thyroid block was given saline (.5 ml.). All animals were killed 20–24 hours after the last injection. The inhibitory effect of thyroid hormone on TSH secretion was similarly tested in 2 groups of non-goitrous rats. One group was given 10 μg of triiodothyronine daily, for 10 days, to the end of the experiment. Rats of the other group received the same hormone treatment but were continued for 10 additional days on saline injections before sacrifice. The approach in the second experiment was similar except in two respects. Duration of PTU treatment before withdrawal

[2] Presented at the 41st meeting of the Endocrine Society, Atlantic City, New Jersey, 959, and at the Thyrotrophin Work Conference, Harriman, New York, 1961.

approximated one year and sodium L-thyroxine (T_4; 10 μg, 10 days) was injected instead of triiodothyronine.

Blood was obtained by cardiac puncture under light ether anesthesia just prior to killing. Pooled sera from rats of each group were frozen until ready for TSH assay. Anterior pituitary extracts were made by homogenization and extraction of 6–10 adenohypophyses in acidified saline and were assayed for TSH in the stasis tadpole (7). Test animals (Rana clamitans) received an extract equivalent of .1–.2 mg. of fresh pituitary or .25–.30 ml. serum over a 4–5 day injection period. At least 3 dose levels of the U.S.P. Reference Standard were tested in parallel bioassay experiments. TSH content of sera and pituitary was estimated from the standard log-dose response curve and the data so obtained were subjected to an analysis of variance. At autopsy, the thyroid and other endocrine glands were quickly and cleanly dissected, weighed to .1 mg. on a torsion balance and prepared for microscopic study. The cellular structure of the adenohypophysis was evaluated after Helly or Susa fixation (Halmi modification) on paraffin sections cut serially at 3 micra through the horizontal plane. Masson staining was used for routine differentiation of cellular types in the pars distalis. Identification of the basophilic varieties was done with the periodic acid-Schiff (PAS) method and the aldehyde-fuchsin (AF) staining technic of Gomori. All data were evaluated statistically. Standard error of the mean was calculated from the formula $S.E. = \sqrt{Ed^2/N(N-1)}$. Fisher "T" tables were used to assess levels of significance.

RESULTS

Pituitary-thyroid function with chronic PTU treatment

TSH levels in sera were found to be relatively constant in both young and old untreated normal rats. Mean concentrations ranged from 67 to 100 mu. per 100 ml. of serum with a consistent tendency for higher values in old animals (Table 1; Groups F–H). The average concentration for all

TABLE 1. TSH CONTENT OF SERUM AND ADENOHYPOPHYSIS IN NORMAL AND GOITROUS RATS

Group	No. of animals	Mean body weight (g.) ±S.E.	Days on PTU	Mean gland weight Mg./100 gm. body		TSH assay		
				Thyroid ±S.E.	Adenohypophysis ±S.E.	Serum mU./100 ml. ±S.E.	Adenohypophysis* mU./mg. ±S.E.	Adenohypophysis* mU./gland ±S.E.
Normal:								
A	10	204 ± 15		7.0 ± .4	4.5 ± .3	80 ± 12	85 ± 23	782 ± 51
B	9	202 ± 12		6.6 ± .3	3.2 ± .3	70 ± 10	50 ± 13	325 ± 30
C	12	231 ± 10		7.4 ± .3	4.0 ± .3	67 ± 8	55 ± 18	506 ± 38
D	17	253 ± 17		7.1 ± .2	3.7 ± .2	70 ± 7	100 ± 20	936 ± 50
E	30	247 ± 8		6.2 ± .2	4.4 ± .2	—	66 ± 30	726 ± 33
F	11	270 ± 10		6.1 ± .5	4.3 ± .3	100 ± 11	70 ± 15	875 ± 56
G	15	320 ± 9		5.4 ± .2	3.8 ± .2	100 ± 8	15 ± 5	182 ± 9
H	11	376 ± 12		6.8 ± .4	4.1 ± .2	90 ± 10	4 ± 1	60 ± 3
PTU:								
I	12	213 ± 15	18	19.8 ± 1.0	4.6 ± .3	130 ± 20	12.0 ± 5.0	118 ± 8
J	10	204 ± 8	20	29.3 ± 2.0	4.8 ± .4	150 ± 17	2.4 ± .3	24 ± 2
K	19	251 ± 25	30–35	25.7 ± 3.0	4.7 ± .2	120 ± 20	2.3 ± .8	28 ± 2
L	19	255 ± 10	50–60	34.1 ± 2.5	4.6 ± .2	160 ± 20	1.0 ± .1	14 ± 1
M	14	262 ± 10	90–130	46.5 ± 4.0	6.6 ± .4	240 ± 38	3.0 ± 1.0	54 ± 3

* Mean TSH concentration and content of the "average" adenohypophysis (wet weight) expressed in U.S.P. mU.

TABLE 2. TSH LEVELS IN SERUM AND ADENOHYPOPHYSIS AFTER PTU WITHDRAWAL

Group	Days off PTU	Mean gland weight (Mg./100 gm. body		TSH assay	
		Thyroid ±S.E.	Adenohypophysis ±S.E.	Serum TSH/100 ml.	Adenohypophysis TSH/gland
				(mu.-U.S.P. ±S.E.)	
N*	0	29.1 ± 3.2	4.8 ± .2	130 ± 15	28 ± 2
O	3	27.4 ± 2.5	4.8 ± .3	—	14 ± 1
P	7	16.3 ± 1.1	4.2 ± .3	<40	3294 ± 216
Q	12	11.9 ± .9	3.9 ± .3	60 ± 10	2225 ± 200
R	20	8.5 ± .4	4.9 ± .4	100 ± 12	1440 ± 110

* All groups (10–12 rats each) were maintained on PTU for 30–35 days before withdrawal of the goitrogen.

normal groups combined was 82.4 ± 9.4 mu. of U.S.P. Reference Standard per 100 ml. of serum. Hormone concentrations in the adenohypophysis were more variable. The mean pituitary concentration in rats from 4–8 months of age (Groups A–F) was 71 ± 17 mu. per mg. TSH content of the pituitaries averaged 690 ± 40 U.S.P. mu. per gland (TSH concentration × mean fresh pituitary weight ± standard error of the mean). Adenohypophysial TSH levels in old rats (Group H) fell to less than 10% of this value although circulating hormone titers were well maintained. The relationship of gland to body weight with advancing age was not appreciably altered for either thyroid or pituitary.

Continued propylthiouracil treatment produced progressively larger goiters and eventually significant pituitary hypertrophy (Table 1). Serum TSH levels were consistently elevated and were maintained with relatively little fluctuation up to 60 days of treatment with the drug. Mean TSH concentration in serum over this period was 140 ± 9 mu. per 100 ml. Longer treatment with PTU resulted in further increase (Group M). Regardless of TSH blood level or size of the goiter in PTU-treated rats, hormone content of the pituitary was invariably reduced. Depletion of pituitary TSH stores was almost complete after 1–4 months on the goitrogen; total hormone reserves in the gland were usually less than 5% of normal. The cytological picture in the pars distalis was characterized by virtually complete disappearance of the acidophils and an increase in numbers of large multi-vacuolated basophils with little or no AF-positive granulation (thyroidectomy cells).

Effect of goitrogen withdrawal on the pituitary-thyroid system

The sequence of change in pituitary and serum TSH levels after goitrogen withdrawal is shown in Table 2. In goitrous rats (30–35 days on PTU), release from thyroid block induced substantial accumulation of thyro-

trophin in the adenohypophysis. The effect was not fully apparent until 6–7 days after cessation of PTU treatment. Pituitary TSH stores were still depressed a few days after goitrogen withdrawal although cytologic examination of the adenohypophysis at this time indicated return of some glycoprotein granulation in the cytoplasm of the thyroidectomy cells. At the peak of rebound, AF-positive, vacuolated basophils in the pars distalis were numerous (Figs. 1 and 2). TSH stores had increased to about 500% of normal but serum titers at this time were subnormal. Hormone concentrations in the pituitary were still augmented 20 days after release from PTU although circulating levels had achieved the normal range. Many thyroidectomy cells were still visible in the pars distalis at this time. Goiters were gradually reduced in size but the thyroid was still enlarged at the end of the experiment. TSH rebound was not reflected in any change in weight of the adenohypophysis.

Exogenous Thyroid Hormone and TSH Rebound

Several major findings emerge from the experiments with thyroid hormones (Fig. 3). 1) Vigorous TSH rebound in the pituitary still occurred and was possibly intensified after 1 year of PTU treatment. The amplitude of the response at the same time interval (10 days) was apparently greater in the hypertrophied pituitary gland. 2) A dose of triiodothyronine which resulted in almost complete disappearance of thyrotrophin in both blood and pituitary of the euthyroid animal was only partially effective in suppressing hormone accumulation in the gland after goitrogen withdrawal. On an equal weight basis, thyroxine was much less effective than triiodothyronine in preventing TSH repletion in the pituitary. That the latter is a more potent inhibiting agent on pituitary TSH secretion has already been shown (5, 23). 3) Marked diminution in level of thyrotrophin in the hypophysis (and blood) with triiodothyronine was not followed by exaggerated hormone accumulation in the gland after thyroid treatment was discontinued. Blood TSH titers remained subnormal. This suggested that the lowered content of pituitary TSH per se was not primarily responsible for the rebound phenomenon. 4) The course of blood TSH levels in goitrous rats after exogenous thyroid hormone administration was erratic. Serum concentrations in hyperthyroid rats decreased to normal or subnormal values without apparent relation to the quantity of thyrotrophin in the pituitary.

DISCUSSION

Even though rates of formation and release of trophic hormones cannot be measured directly, a reasonably accurate analysis of the dynamics of TSH secretion can be made if thyrotrophin levels in pituitary and blood and rate of disappearance of the hormone from the circulation are known. Recovery of intravenously administered heterologous and homologous

Explanation of Figs. 1 and 2

Photomicrographs of comparable areas in the pars distalis of the normal rat and in the goitrous animal during TSH rebound.

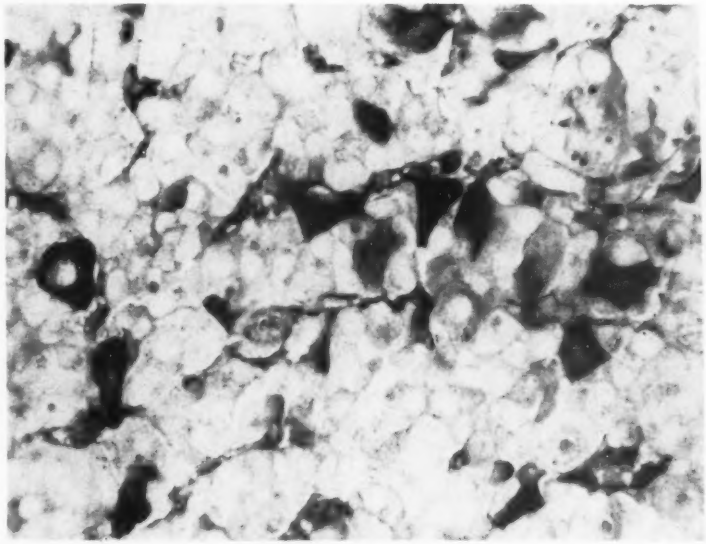

Fig. 1. The angular thyrotrophs of the normal are shown (black) selectively stained with basic aldehyde fuchsin. The cytoplasmic granulation is flocculent and dense. Nuclei and other cell types are feebly and non-specifically stained. (Helly; Gomori's Paraldehyde Fuchsin, ×750.)

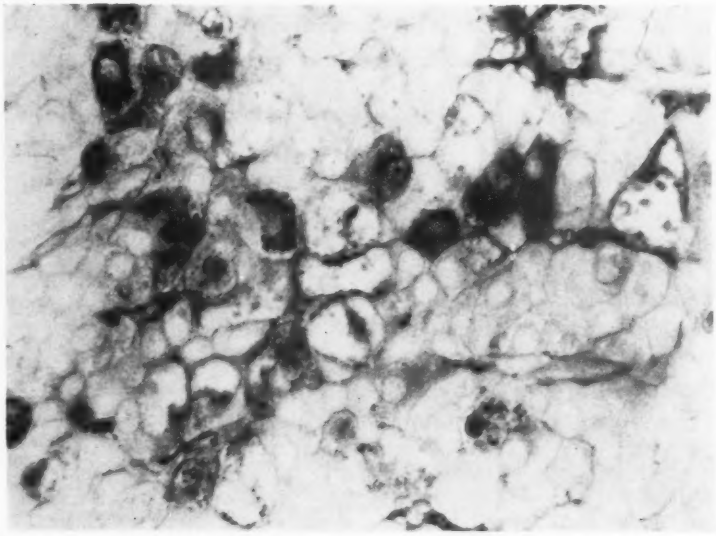

Fig. 2. Reaccumulation of AF-positive cytoplasmic material is shown in a cluster of thyroidectomy cells during the rebound phase 8 days after cessation of PTU treatment (135 days). The granules are found in the peripheral cytoplasm outside the vacuoles. Vacuolar contents are not fuchsinophilic. (Susa; Gomori's Paraldehyde Fuchsin, ×750.)

thyrotrophin from rat plasma has been determined with close agreement (8–12). Rate of disappearance of hormone from the blood is rapid in initial stages but slows progressively as physiologic levels are approached. An approximate estimate of the half life of TSH in the circulation of the euthyroid rat would be at least one hour.[3] If it is assumed that this half life describes endogenous TSH disappearance from plasma as well, it can be calculated that the adenohypophysis of the adult female rat normally possesses the equivalent of several days supply of thyrotrophin. That this

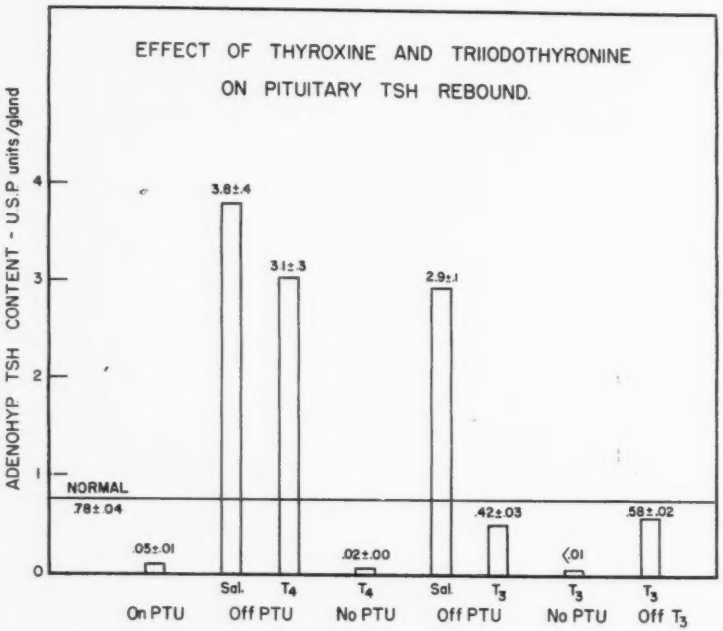

FIG. 3. Height of the bar depicts the mean TSH content ± standard error of the mean of the "average" pituitary expressed in U.S.P. units. The horizontal line gives the normal control value in this set of experiments. TSH rebound was tested 10 days after goitrogen withdrawal (off PTU) or cessation of thyroid treatment (off T_3). T_3 = triiodothyronine; T_4 = thyroxine; No PTU = no previous goitrogen treatment.

is at best a tentative value needs to be emphasized. The metabolic fate of TSH is unknown and the hormonal equilibrium which exists between

[3] It is calculated from data already given (8, 9) that the relationship between hormone recovery (R) and time (T) is described by the equation Log R = 1.965 − 0.860 log T. The slope represents change in Log R per unit change in Log T. The biological half life, or time to effect a 50% reduction in hormone recovery (R1 to R2), = antilog 0.35 or 2.2 × the number of minutes (T1) already elapsed since introduction of hormone into the circulation.

the vascular and extracellular fluid compartments and the tisuses has not been satisfactorily delineated.

It is evident that in the goitrous rat the combined amount of thyrotrophin in blood and pituitary after propylthiouracil release is far in excess of that present in any other endocrine state so far described. The amplitude of change in hormone levels before and after goitrogen withdrawal indicates that thyrotrophin secretion is markedly asynchronous during rehabilitation of the pituitary-thyroid system. Augmented hormonal stores in the adenohypophysis cannot be explained on the basis of normal synthesis rate with decreased release. Pituitary rebound co-exists with normal or elevated thyrotrophin levels and occurs at a time when plasma turnover must be increasing rather than diminishing (9). Cytological evidence fully supports the view that synthesis and storage of TSH by the pituitary during the rebound phase are accelerated without commensurate augmentation in release. Previous studies have demonstrated excellent correlation between the numbers of AF-positive, polygonal basophils (beta cells, thyrotrophs) in the pars distalis and the functional status of the pituitary-thyroid system (13, 14, 4, 5). These cells are numerous and their glycoprotein granulation abundant when TSH stores in the pituitary are high. After surgical or chemical thyroidectomy, the thyrotrophs lose their cytoplasmic granulation and transform into hypertrophied, multi-vacuolated basophils (goiter or thyroidectomy cell); TSH reserves in the adenohypophysis diminish substantially. The maintenance of elevated blood thyrotrophin levels in goitrous animals for periods even exceeding one year and the return of AF-positive granulation in the basophils during TSH rebound substantiate the belief of others that the thyroidectomy cell is a highly active secretory unit (13–15) and that the cytoplasmic granulation of the beta basophil is the morphological expression of thyrotrophin storage.

It would appear that the mechanisms underlying the TSH rebound phenomenon must be humoral rather than neural. D'Angelo and Traum (4) and Florsheim (16) demonstrated that exogenous thyroid hormone suppresses pituitary TSH secretion equally well in normal and hypothalamic lesioned rats. The capacity of the adenohypophysis for TSH repletion is also unimpaired after hypothalamic destruction (5). The finding that normally inhibiting doses of thyroid hormones are relatively ineffective in preventing TSH rebound after goitrogen withdrawal strongly suggests that altered sensitivity of the pituitary to a target gland hormone may occur independently of any neural influence. This result serves to re-emphasize the fact that the operation of the feedback mechanism between thyroid and hypophysis is not under hypothalamic domination and that the basal level of TSH function in the "isolated" pituitary is not fixed.

Knowledge of biochemical events governing thyrotrophin secretion is sparse and provides no clue as to the secretory dynamics of the rebound phenomenon. Hypersecretion of TSH in the thyroidectomized rat is not

associated with significant change in enzyme levels (cytochrome oxidase, succinic dehydrogenase) although *in vitro* oxygen consumption of the pituitary increases (17). S^{35}-labeled methionine uptake of the pituitary decreases in cold-exposed rats (18) but is not materially altered during TSH rebound in goitrous animals (unpublished experiments). There is reason to believe that the functional activity of the unblocked thyroid gland may be involved in adenohypophysial thyrotrophin rebound inasmuch as the thyroid itself exhibits temporary dichotomy in function after release from propylthiouracil. D'Angelo and associates (19) reported enhanced uptakes of I^{131} by the guinea pig thyroid which persisted for two weeks after goitrogen withdrawal; other parameters of thyroid function returned slowly to normal without rebound. The iodide pump activity per mg. of thyroid in the mouse increased 24–48 hours after PTU release even though morphologic regression occurred (20). Shimoda (21) described augmentation of thyroidal radioiodine uptake and conversion ratio in the rat 7 days after goitrogen withdrawal. Interestingly, coincidental rebound of neurosecretory substance in the neurohypophysis was also observed. It appears likely that upon release from thiouracil block the sequential processes of iodide concentration and hormonal synthesis and release by the thyroid, like TSH secretion by the pituitary, are temporarily asynchronous.

The TSH rebound phenomenon can be explained by assuming a differential threshold type of thyroid hormone action. A low circulating level of newly formed thyroid hormone from the unblocked gland may provide the stimulus to the pituitary for heightened synthesis and storage during that period when the high momentum of thyrotrophin secretion "overshoots" (after goitrogen withdrawal). The TSH release phase is not equally affected so that superabundance of hormone in the pituitary with normal or reduced amounts in blood results.[4] As the thyroid gland gradually regains the steady state, blood levels rise sufficiently to limit the production and storage of thyrotrophin. Further increase in thyroid hormone to excessive levels (for example, exogenous hormone administration) depresses both synthetic and release phases of TSH secretion and hormone content of both blood and hypophysis would be reduced. The fact that thyrotrophin rebound did not occur after pituitary stores were virtually depleted with triiodothyronine suggests that a state of TSH hypersecretion must first be induced before the effect can be elicited. This differential threshold hypothesis is being tested by surgical removal of thyroids from goitrous animals and by thyroxine or triiodothyronine replacement therapy.

[4] This may possibly account for those relatively few reports which claim that pituitary TSH content in the surgically or chemically thyroidectomized rat is increased. A functional thyroid remnant or incomplete block with a thiouracil would permit slight yet effective thyroid hormone secretion. In some rodents, e.g., the guinea pig, TSH levels in the hypophysis rise considerably with continued goitrogenesis (22).

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OVARIAN ASCORBIC ACID AS AN ENDOGENOUS AND EXOGENOUS ASSAY FOR CYCLIC PROESTROUS LH RELEASE[1]

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ABSTRACT

Cyclic proestrous LH release was investigated by endogenous and exogenous assay in rats exposed to light from 5:00 A.M. to 7:00 P.M. daily. Pituitaries were removed from proestrous rats at 10:00–11:00 A.M. and 4:00–5:00 P.M., injected into immature, primed recipient rats and tested for ovarian ascorbic acid depleting ability (exogenous assay). "Morning" pituitaries were significantly more potent than "afternoon" pituitaries, confirming the finding of drug and hypophysectomy studies that LH is released from the pituitary between 2:00 and 3:00 P.M. Ovaries removed from the proestrous donor rats showed no consistent ascorbic acid depletion on the afternoon of proestrus (endogenous assay). There was, however, a cyclic variation in ovarian ascorbic acid, the highest values occurring in metestrus, the lowest in proestrus.

IN RECENT years Everett and his co-workers (1, 2) have accumulated indirect evidence suggesting that when cyclic female rats are exposed to light daily between 5:00 A.M. and 7:00 P.M., LH is released from the pituitary starting at 2:00 P.M. and completed in 90% of animals by 3:30 P.M. on the day of proestrus. Ovulation then occurs around midnight. This timing of LH release was demonstrated by injecting a variety of centrally-acting blocking drugs (1) or hypophysectomizing (2) at the critical time in the afternoon and looking for presence or absence of ovulation the following morning.

The use of endogenous ovulation as a criterion of LH release necessitates the presence of prepared follicles, limiting the variety of experiments which might be utilized to elucidate the factors triggering LH release. In order to avoid the need for a prepared ovary, changes in LH content must be measured directly, either in the anterior pituitary or in the body fluids. Acute decreases in pituitary gonadotrophin content have been demonstrated following coitus-induced ovulation in the rabbit (3, 4), following the onset of heat in the ewe (5) and sow (6), and following post-partum ovulation in the mouse (7). This encouraged us to look for acute decreases

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in pituitary LH content in the cyclic rat following the 2:00–3:30 P.M. release period, by removing pituitaries from adult cyclic proestrous rats at two times of day spanning this period (10–11 A.M. and 4–5 P.M.). These pituitaries were then injected into primed, immature assay rats for the exogenous measurement of LH content by the ovarian ascorbic acid depletion technique (8).

As a matter of secondary interest, the donor proestrous animals were also tested endogenously for LH release, by measuring the ascorbic acid content of their own ovaries, removed simultaneously with the pituitaries. This is analogous to techniques utilizing adrenal ascorbic acid for determining stress-induced ACTH release. As is well known, following a stress ACTH release can be detected by either an endogenous depletion of adrenal ascorbic acid in the stressed animal (9) or by a drop in pituitary ACTH content, using as an assay of ACTH the depletion of adrenal ascorbic acid in a recipient, hypophysectomized rat (10). In the case of ACTH, the endogenous assay appears to be a more consistent indicator of acute ACTH release than the exogenous assay (11).

MATERIALS AND METHODS

Pituitary Collection and Assay (Exogenous)

Adult Cyclic Donor Females: Pituitary Collections. Ninety-day-old virgin female rats were shipped from Sprague-Dawley, Inc., in 4 lots of 50 rats each and placed in a constant temperature (25° C) room which was artificially lighted from 5:00 A.M. to 7:00 P.M. Vaginal smears were taken each morning and every rat which served as a pituitary donor on the day of proestrus had run at least two previous consecutive four-day cycles before the cycle during which it was autopsied. We have previously confirmed by pentobarbital blockade that LH release occurs between 2:00 P.M. and 4:00 P.M. on the day of proestrus in the Sprague-Dawley rat under the above lighting conditions (12). Accordingly, animals were decapitated between 10:00 and 11:00 A.M. ("morning") or 4:00 and 5:00 P.M. ("afternoon") and the pituitaries removed immediately. The neurohypophysis was dissected free and discarded. The anterior pituitaries were either frozen immediately and homogenized on the day of the assay, or immediately homogenized, suspended in saline and kept frozen for assay a few days later. Five morning and five afternoon pituitaries were pooled for use in each group of assay rats, a total of 40 morning pituitaries and 40 afternoon pituitaries being collected.

Immature Primed Recipient Females: Pituitary Assays. The method of assay was that of Parlow (8, personal communication). The recipient females were received as weanlings in 8 lots from Sprague-Dawley, Inc., and pre-injected subcutaneously with 50 I.U. PMS (Gonadogen, Upjohn) when 22 days old and 25 I.U. HCG (Antuitrin "S," Parke, Davis) 56–60 hours later. They were used for the assay of pituitaries on the 7th day after the HCG injection. On that day two pools of five pituitaries were diluted to yield concentrations ranging from 1/64 to 1/2 of a pituitary equivalent per 0.5 cc. of saline. In each of the 8 assays, both a morning and an afternoon pool of proestrous pituitaries was used, at two or three dose levels. Each recipient rat was anesthetized with 4 mg./100 gm. pentobarbital. The left ovary was removed for a control. Then 3 to 6 recipient rats received 0.5 cc. of a given dose of pituitary (or control saline) via the tail vein. One hour later the right ovary was removed. Both ovaries were dissected free of fat, weighed, and ascorbic acid measured by the method of Roe and Kuether (13).

Adult Ovaries: Collection and Ascorbic Acid Measurements (Endogenous Assay)

Ovaries were removed for ascorbic acid measurement at the time of pituitary removal from 42 of the above adult proestrous animals, and also at the same two times of day from 48 four- or five-day cyclic metestrous, estrous and diestrous rats received and housed simultaneously (these 90 rats are designated as "A" series below). Subsequently, this experiment was repeated in another group of 20 adult rats housed similarly and autopsied at 10–11 A.M. and 4–5 P.M., ovaries again being removed for endogenous ovarian ascorbic acid concentration measurements in four cycle stages ("B" series). Presence of ova in the oviducts was verified for all estrous rats in both series. Diestrus was defined as the day after metestrus in the A series, and the day before proestrus in the B series. Ascorbic acid was measured in A ovaries by the method of Roe and Kuether (13). However, ascorbic acid measurements in B ovaries was carried out using the much more convenient, if less sensitive, technique of Mindlin and Butler (14).

A preliminary study was carried out comparing the two methods used to measure ascorbic acid in the adult ovaries. Ten adult female rats were decapitated and the ovaries and adrenals removed bilaterally, trimmed and weighed. Ascorbic acid was measured in one adrenal and ovary from each rat by the method of Roe and Kuether (13); ascorbic acid in the other adrenal and ovary was measured by the method of Mindlin and Butler (14). For the Roe and Kuether method, the adrenal ascorbic acid was $4.04 \pm .75$ (μg/mg.), the ovarian ascorbic acid was $0.72 \pm .26$. For the Mindlin and Butler method, adrenal ascorbic acid was $4.17 \pm .30$, ovarian ascorbic acid was $.77 \pm .20$. There is no significant difference between the two methods for either gland.

RESULTS AND DISCUSSION

Exogenous Proestrous Pituitary Assay. Each of the five doses of pituitary (1/64, 1/32, 1/16, 1/8, 1/2) was tested for ovarian ascorbic acid depleting ability in at least two assays. The depletion at each dose was corrected for the depletion in recipients simultaneously injected with saline. The net result of saline injections (in all 8 assays) was to yield insignificant depletion (Table 1). An analysis of variance was performed (separately for morning and afternoon at each dose) in order to detect possible differences among the assays of the same dose of similar materials. Only in the case of the 1/2 pituitary dose (afternoon) was there a significant difference among assays. Accordingly the results of all assays performed at each dose level were pooled for subsequent analyses. These are summarized in Table 1. The method was sufficiently sensitive to detect hormone in as little as 1/64 of a morning pituitary, and 1/32 of an afternoon pituitary. There is a highly significant dose-response effect of pituitaries removed at either time of the day, with the plateau occurring between 1/16 and 1/8 of a pituitary (Table 1). An analysis of variance utilizing all of the doses, as previously reported (15), fell short of significance in demonstrating a difference in depleting ability between morning and afternoon pituitaries because of the inclusion of the plateau data. However, when data from the plateau region are eliminated (1/8 and 1/2 doses), an analysis of variance reveals a highly significant difference in depleting ability between morning and afternoon pituitaries, the morning pituitaries being more potent. We have since verified that this diurnal drop in LH content is unique to the

TABLE 1. OVARIAN ASCORBIC ACID DEPLETING ABILITY OF PROESTROUS "MORNING" AND "AFTERNOON" PITUITARIES

Dose of pituitary used	"Morning"			"Afternoon"		
	No. of assays	No. of recipient animals	% ovarian* asc. acid depletion	No. of assays	No. of recipient animals	% ovarian* asc. acid depletion
None (Saline Injection)	8	40	1.07 ±1.10†			
1/64	3	12	12.58 ±2.99	3	13	4.30 ±2.75
1/32	4	20	19.55 ±1.72	4	19	15.89 ±1.99
1/16	2	8	25.87 ±2.00	2	8	19.41 ±4.38
1/8	3	15	29.40 ±4.42	3	15	30.73 ±2.58
1/2	4	16	33.43 ±2.05	4	16	32.31 ±1.62

* $\frac{\text{(Asc. Acid, } \mu\text{g./mg. Left)} - \text{(Asc. Acid, } \mu\text{g./mg. Right)} \times 100}{\text{Asc. Acid } \mu\text{g/mg. Left}}$

For pituitary injections, the % depletion for each was corrected for the depletion of the saline injected rats of the same assay.

† ±Standard error.

day of proestrus, and does not occur on any other day of the cycle (16).

Thus, the pituitary of the proestrous rat, like that of the estrous ewe (5) and sow (6), post-partum mouse (7) and post-coital rabbit (3, 4) shows a drop in luteinizing hormone content following hormone release. Therefore, it is possible to use pituitary LH content as measured by ovarian ascorbic acid depletion as an indicator of acute hormone release instead of observing resulting ovulation 10 to 12 hours later (1). Blocking evidence indicated that the stimulus for LH release starts at 2:00 P.M., with enough hormone being released by 3:30 P.M. to induce ovulation (2); the present evidence indicates that enough had been released by 4:30 P.M. to cause a significant drop in pituitary stores. The possibility that a decrease in the rate of manufacture of hormone contributed to this drop cannot be eliminated on the basis of the present study.

Previous studies of gonadotrophin content of the cyclic rat pituitary did not take cognizance of the 2–3:30 P.M. critical period on the day of proestrus. Gans and De Jongh (17), measuring the ICSH content of injected pituitary material (by increase in prostate and testicular weight of immature recipients), found a higher content of ICSH the day before proestrus than at any other stage. Measuring ICSH by implantation (rather than injection) into recipients shifted the peak pituitary potency to the day of proestrus. Lane and Hisaw (18) also measured LH by pituitary implantation in recipents and claimed a higher LH content in estrus than diestrus. It is difficult to interpret either of the afore-mentioned studies in the absence of information concerning animal lighting conditions, and autopsy times.

Santolucito and co-workers (5) obtained a significant drop in pituitary FSH at the same time (35 hours) as LH dropped in the ewe following the first appearance of heat. Since Parlow (8), McCann and Taleisnik (19) and Schwartz (unpublished observations) found that FSH gives no depletion of ovarian ascorbic acid except in doses leading one to suspect LH contamination, the drop in ascorbic acid depleting potency on the afternoon of proestrus cannot be the result of a drop in FSH content. However, since the assay does not measure FSH, we cannot rule out the possibility that FSH, as well as LH, potency decreases on the afternoon of proestrus in the rat.

Endogenous Ovarian Ascorbic Acid. Ovarian weights and ascorbic acid concentrations are shown for both A and B series in Table 2. Ovarian weights were similar in the two series. Both show a rise from proestrous morning to estrous morning or afternoon. Otherwise no trend is obvious.

Ascorbic acid concentrations were systematically higher in series A than B. However, both show a rise from proestrus to metestrus (maintained on diestrus day 1 for series A), and then a decline back to proestrus. In the large series of rats in the A series no evidence of ascorbic acid depletion occurred on the day of proestrus, in spite of LH release (Table 1). However, there is a significant drop ($P < .01$) in the B ovaries during this day. The results of the endogenous assay therefore appear equivocal.

In retrospect, it is not surprising that endogenous ovarian ascorbic acid depletion failed to occur consistently (Series A, Table 2). The only demonstrations of ovarian ascorbic acid depletion following acute gonadotrophin administration have been obtained from ovaries containing functional corpora lutea: gonadotrophin primed immature (8, 19) or mature rats (19); pregnant rats (20) or rabbits (21). Moreover, the adult rat, even when primed with gonadotrophin, is considerably less sensitive than the immature primed rat (19), so that negative results were perhaps to be expected in insensitive unprimed adult rats. The positive results seen in Series B could mean that, in spite of relative insensitivity, these rats may show ovarian ascorbic acid depletion in response to acute increases of circulating LH; this is analogous to the adrenal ascorbic acid depletion following acute ACTH release in unprimed rats (9). However, an alternative interpretation may be offered. It will be noted in Table 2 that in both series ovarian ascorbic acid falls from a maximum at metestrus to a minimum at some time on the day of proestrus. Hoch-Ligeti and Bourne (22) found that ovarian ascorbic acid was maximum at metestrus and diestrus, began falling in proestrus and reached a minimum on the day of estrus (no autopsy times stated). It is possible that the proestrous drop in Series B is just part of the general picture of diminishing ascorbic acid concentration between metestrus and the following proestrus or estrus and is not the result of the 2:00 P.M. LH release.

It seems premature to hypothesize any specific gonadotrophin control

TABLE 2. OVARIAN WEIGHT AND ASCORBIC ACID CONCENTRATION IN DONOR CYCLIC RATS

Source		Ovarian weight, mg./100 gm B.W.		Ascorbic acid μg/mg. ovarian weight	
Cycle stage	Time of day	A	B	A	B
Proestrus	Morning	28.8 ± 3.2* (20)	30.0 ± 4.2 (5)	0.77 ± .11 (20)	0.68 ± .07 (5)
Proestrus	Afternoon	29.4 ± 3.2 (22)	33.9 ± 3.1 (5)	0.76 ± .14 (22)	0.50 ± .07 (5)
Estrus	Morning	30.8 ± 4.8 (12)	34.8 ± 4.3 (5)	0.82 ± .06 (12)	0.69 ± .06 (5)
Estrus	Afternoon	32.9 ± 3.1 (13)	31.1 ± 3.9 (5)	0.89 ± .09 (13)	0.70 ± .11 (5)
Metestrus	Morning	30.1 ± 1.5 (3)	30.3 ± 2.6 (5)	0.93 ± .30 (3)	0.75 ± .14 (5)
Metestrus	Afternoon	29.0 ± 6.1 (5)	31.8 ± 4.4 (5)	0.95 ± .21 (5)	0.84 ± .08 (5)
Diestrus†	Morning	32.2 ± 6.3 (5)	—	0.85 ± .15 (5)	—
Diestrus†	Afternoon	29.7 ± 3.1 (10)	—	0.91 ± .11 (10)	—
Diestrus‡	Morning	—	30.2 ± 1.6 (5)	—	0.69 ± .06 (5)
Diestrus‡	Afternoon	—	31.4 ± 3.4 (5)	—	0.64 ± .02 (5)

( ) No. of rats.
* Standard deviation.
† Defined as the day after metestrus (5-day rats).
‡ Defined as the day before expected proestrus (4- or 5-day rats).

of the cyclic variation (Table 2) in ascorbic acid. Ascorbic acid appears to follow the same course as ovarian lipids (23, 24), which are minimal at proestrus, and reach a maximum on the day of diestrus of 4-day cycles or diestrus day 2 of 5-day cycles. Both lipids and ascorbic acid are correlated, in turn, with ability to form functional corpora lutea when pituitary transplants are made to the kidney capsule (25): such corpora lutea will be produced at estrus, metestrus and early in diestrus, but not late in diestrus or in proestrus when luteolysis is occurring (23–25). In view of this correlation, and the demonstration in the cow that ascorbic acid concentration is much higher in corpora lutea than in the rest of the ovary (26), it is tempting to conclude that the source of cyclic variation in ascorbic acid is the set of corpora lutea from the previous ovulation. Histochemical evidence, however, does not support this interpretation, since Hoch-Ligeti and Bourne (22) found an increased ascorbic acid at metestrus only in the germinal epithelium, and Deane (24) reports an increased ascorbic acid at metestrus, in the corpora lutea not from the most recent ovulation, but the ovulation one cycle before. Moreover, Claesson, *et al.* (21) induced

ascorbic acid depletion in the non-corpus luteal tissue of the pregnant rabbit ovary by PMS injection. In view of these contradictory data, it is difficult to draw any unequivocal conclusions regarding the source within the adult ovary of cyclic variations in ascorbic acid.

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THE INFLUENCE OF AGE ON SUPEROVULATION IN THE IMMATURE RAT AND MOUSE

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ABSTRACT

Studies on superovulation in the immature rat and mouse indicated that the average number of ova released following treatment with gonadotropins reached a maximum on 23 to 32 days of age for the former and 23 to 26 for the latter. Thereafter the number of eggs released decreased rapidly by 50 to 65% prior to puberty although the gonadotropin treatment was held constant. Treatment with higher dosages of gonadotropins (PMS+HCG) at 40 to 50 days of age induced the release of a larger number of ova leading to the conclusion that the decrease in the number of ova released with age was due to changes in the sensitivity of the follicles to the gonadotropins. It would appear that initially the follicles become competent to respond to the gonadotropins at approximately 14 days of age for the mouse and 17 to 18 days of age for the rat. The sensitivity increases to a maximum within two weeks and decreases thereafter.

IMMATURE animals have been frequently used in place of hypophysectomized animals for the assay of various gonadotropins. In general, the immature animal has given results indicative of a lack of endogenous gonadotropin. Recently the phenomenon of superovulation has been utilized for the assay of gonadotropins using immature rats at 21 to 22 days of age (1) and in the course of the investigation we noted marked variations in ova count depending on the age of the animal. This led to the present investigation which is concerned with the influence of age on release of ova following treatment with exogenous gonadotropin.

MATERIAL AND METHODS

Approximately 200 Swiss mice and 200 Wistar rats were used in this study. These animals were taken from the Purdue colony and raised under standard conditions in our laboratory. The mothers were isolated shortly after mid-pregnancy and the date of parturition recorded. All litters were sexed and reduced to 6 within 24 hours after delivery and for this study the young males were preferentially discarded whenever the litter size was greater than 6. In the initial study on age, the mice were injected subcutaneously with 10 I.U. of PMS in a volume of 0.1 ml. saline followed in 40 hours by

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5 I.U. of HCG[3] also given subcutaneously in a volume of 0.1 ml. saline (2). The rats were treated with 30 I.U. of PMS subcutaneously in 0.1 ml. saline followed in 56 hours by a dose of 10 I.U. of HCG subcutaneously and also dissolved in a 0.1 ml. volume of saline (1). Both the mice and rats were killed 20 to 24 hours after the injection of the ovulatory substance, i.e., HCG in this instance, the ova removed from the oviduct and counted under a dissecting microscope (1, 3). The age of the animal recorded in these experiments was the age at the time of the initial injection, i.e., the PMS.

The second set of experiments was carried out according to the above design except the mice were all 40 days of age at the start of the experiment and the rats were all 50 days of age. In addition, the amounts of PMS and HCG were varied in an attempt to determine whether a greater number of ova could be released by changing the amount of each hormone.

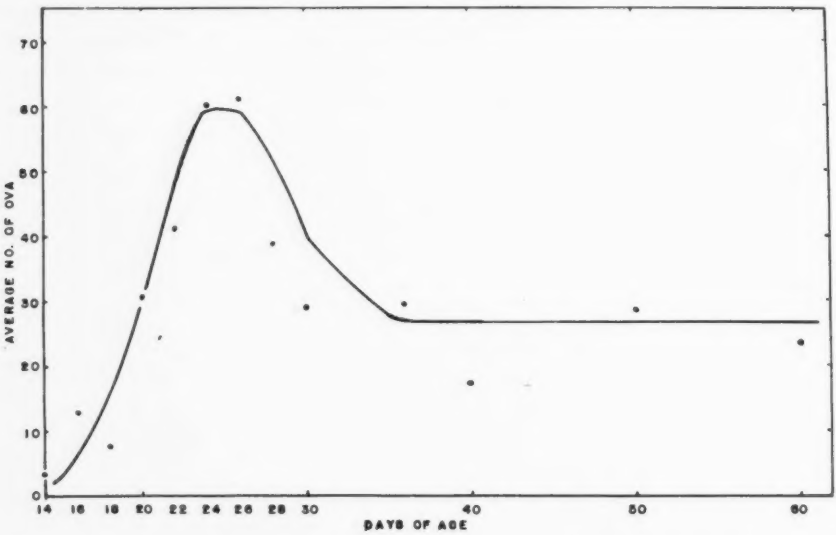

FIG. 1. The effect of age on superovulation in the mouse. The animals were treated with 10 I.U. PMS followed by 5 I.U. HCG.

RESULTS

The influence of age on superovulation in the mouse

In this experiment the dose of PMS and HCG was held constant at 10 and 5 I.U. respectively and the age of the animal varied from 13 to 60 days. At ages of 13–14 days, 40% of the mice ovulated with an average of 3.6 ova per animal (Fig. 1). Approximately 69% of the mice ovulated at ages of 15 to 18 and thereafter the percentage rose to 80% on days 19–20, 93% on days 21–22, and between 86 and 100% thereafter. The average number of ova also rose from 3.6 ova on 13–14 days of age to a maximum

[3] The HCG (APL) and PMS (Equinex) were obtained through the courtesy of Ayerst Laboratories, N. Y. Additional supplies of HCG (Follutein) were obtained through the courtesy of Dr. A. Borman, Squibb Institute, New Brunswick, N. J.

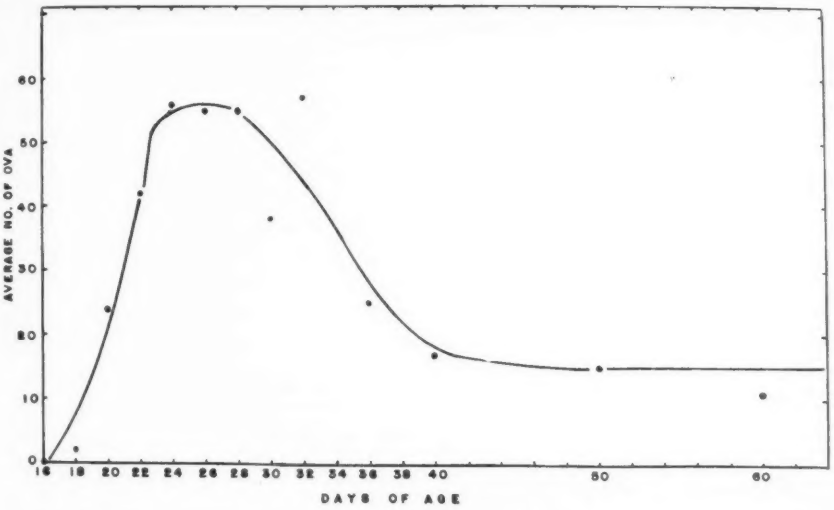

FIG. 2. The effect of age on superovulation in the rat. The animals were treated with 30 I.U. of PMS and 10 I.U. of HCG.

of approximately 60 ova on days 23–26. The ova count thereafter dropped to 29 in animals at 29 to 30 days of age and with minor variations remained unchanged up to 60 days of age. The maximum number of ova recovered from a single mouse was 135 in this experiment.

The influence of age on superovulation in the rat

All rats were treated with 30 I.U. of PMS and 56 hours later given 10 I.U. of HCG. None of the rats treated at 15–16 days of age ovulated; twenty percent ovulated at 17–18 days of age and 75% at 19–20 days of age. One hundred percent of the rats ovulated at 21 days of age and up till 40 days of age. Thereafter the figure dropped to 80% at 49–50 days of age and to 70% at 59–60 days of age. The average number of ova released also showed an initial increase from one ovum per rat at 17–18 days of age to a maximum of approximately 55 ova at 23 to 32 days of age (Fig. 2). Thereafter the count fell to 18 ova on 39–40 days of age although 100% of the animals ovulated. The average number of ova ranged from 13.1 to 18.3 at 40 to 60 days of age. A maximum egg count of 106 was obtained in a rat during the course of this experiment.

The effect of different doses of PMS and HCG on superovulation in the 40–45-day-old mouse

Mice were treated at 40 to 45 days of age with 5 to 40 I.U. of PMS and 1 to 20 I.U. of HCG. Pretreatment with either 10 or 20 I.U. of PMS was adequate to induce superovulation if followed by proper dosages of HCG.

TABLE 1. THE EFFECT OF VARIED DOSES OF PMS AND HCG ON SUPEROVULATION IN 40- TO 50-DAY-OLD MOUSE

No. of mice	PMS I.U.	HCG I.U.	Ova		% mice ovulated
			Average	Range	
5	5	1	13.2	0.32	60
5	5	5	17.8	2–29	100
5	10	1	6.2	0–12	60
5	10	2.5	14.6	0–39	60
5	10	5	18.6	0–37	80
2	10	10	37.5	37–38	100
5	10	20	42.4	29–61	100
4	20	1	0	0	0
10	20	2.5	24.3	1–59	100
10	20	5	27.1	2–74	100
10	20	10	33.1	14–53	100
10	20	20	42.1	13–67	100
5	40	5	4.3	1–9	100

Ten to 20 I.U. of HCG preceded by 20 I.U. of PMS resulted in the average release of 37.5 and 42.4 ova respectively (Table 1). Pretreatment with 20 I.U. of PMS led to a superovulation following treatment with 2.5 to 20 I.U. of HCG. An average number of 24.3 ova was obtained with the lower dose of HCG and 42.1 ova with the higher dose.

The effect of different doses of PMS and HCG on superovulation in the 50-day-old rat

Rats were pretreated at 50 days of age with doses of PMS ranging from 5 to 100 I.U. These doses were followed in 56 hours by various doses of HCG ranging from 2.5 to 20 I.U. (Table 2). An average ova count of 15.1 to 21.4 was obtained with 30 I.U. of PMS and 2.5 to 20 I.U. of HCG; and a count of 15.5 to 19.5 with 50 I.U. of PMS and 5 to 20 I.U. of HCG. No indication of increasing number of ova following increased dosage of HCG was noted in the above treatment. However, pretreatment with 100 I.U.

TABLE 2. THE EFFECT OF VARIOUS DOSES OF PMS AND HCG ON SUPEROVULATION IN 50-DAY-OLD RATS

No. of rats	PMS I.U.	HCG I.U.	Ova		% rats ovulated
			Average	Range	
5	5	10.0	6.2	0–10	80
3	10	10.0	3.0	0– 5	67
5	15	10.0	11.4	9–17	100
2	15	20.0	9.0	7–11	100
5	30	2.5	19.2	0–29	80
5	30	5.0	21.4	11–30	100
10	30	10.0	15.1	0–38	80
6	30	20.0	16.7	7–27	100
10	50	5	15.5	0–31	90
10	50	10	19.5	0–26	90
10	50	20	16.9	8–29	100
10	100	5	12.7	6–20	100
10	100	10	12.6	8–15	100
10	100	20	21.1	12–33	100

of PMS followed by 5 to 10 I.U. of HCG gave an average ova count of 12.7 and 12.6 ova. Increase in the dose of HCG to 20 I.U. resulted in an increase in the average ova count to 21.1.

DISCUSSION

The present results confirm the significance of age in the superovulatory response in both mice (4) and rats. Following treatment with gonadotropins, no ova were obtained in the mice prior to 13 days of age, nor in the rats prior to 17 days of age. The failure of Price and Ortiz (7) to obtain follicular maturation at 18 days of age in the rat treated with PMS is probably due to a strain difference. It is of interest to note that the onset of induced-ovulation is obtained after antrum formation which occurs at 12 to 14 days of age in mice (5) and 11 days of age in rats (6). This would tend to confirm the hypothesis that only follicles with antra are capable of being stimulated and thus the phenomenon may be identified with the developing competence of the follicle to respond to gonadotropins. Once competence to respond is established the response increases rapidly by virtue of an increasing number of follicles becoming capable of responding. Nevertheless the number of ova released at the time of maximum response is still a minute part of the number present in the ovary.

The decrease in number of ova released in the mouse from 27 to 60 days of age as compared to the response at 25 to 26 days of age is explainable on the basis of increased requirement for gonadotropins. Comparable numbers of ova were released at 40 to 45 and at 23 to 26 days of age if the amount of gonadotropin, especially the HCG, was increased. Thus the phenomenon is not one of decreasing number of ova with age (8, 9) since this decrease still allows for the presence of many more ova than are being released but appears to be a situation comparable to sensitivity changes in the reproductive tract of the rat (10). In general the rat behaved similarly to the mouse although the dosages of gonadotropin used at 50 days of age failed to release as many ova as obtained at days 23 to 32. Nevertheless superovulation was induced in the 50-day-old animal.

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EFFECT OF SOY-FLOUR DIET AND OTHER AGENTS ON EXCRETION OF I^{131} AFTER USE OF LABELED THYROXINE IN THE RAT[1]

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ABSTRACT

The fecal and urinary excretion of radioiodine (I^{131}) after injection of radioactive thyroxine was measured in rats fed diets of soy flour, starch-casein, standard laboratory chow and the Remington mixture. Rats fed the soy-flour, starch-casein and Remington diets excreted less I^{131} in the feces than did control rats. No difference was present in the biliary excretion of I^{131} or in the transformation of I^{131}-thyroxine by the intestinal contents. The weight of stools of the rats consuming the test diets was one half that of the control group. The thyroidal uptake of I^{131} and the serum protein-bound iodine (PBI) were low in the rats fed soy-flour diets. The thyroid showed a small increase in weight, but the gland exhibited less cellular activity as compared with the control group.

It was concluded that the fecal excretion of I^{131} after administration of labeled thyroxine is not easily altered. An increase in the bulk of the stools or an increase in biliary excretion of I^{131} allows an abnormally increased loss of I^{131} in the feces. A decrease in weight and bulk of the stools is accompanied by a decreased loss of I^{131}. The small goiter produced in rats fed soy flour did not appear to be on the basis of excessive loss of thyroxine in feces.

VAN MIDDLESWORTH (1) found that rats fed a diet rich in soy flour became goitrous and excreted three times as much radioiodine (I^{131}) in the feces after a tracer dose of radioactive thyroxine as did rats fed a starch-casein "control" diet. This excessive fecal excretion of the thyroxine, or its degradation products, was considered to be the cause of the goiter. Previously, propylthiouracil was the only agent known to cause an increased fecal excretion of I^{131} after use of radioactive thyroxine (2), possibly because of an increased biliary excretion of radioactivity (3).

The implication of Van Middlesworth's work is that soy flour contains or acts like a "thyroxorrheic" agent. Since a goitrogen acting exclusively in this manner had not been described previously, it was thought worth while to pursue further investigation of the effects of a soy-flour diet and other experimental diets, of agents known to influence phases of the entero-

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hepatic circulation of thyroxine, and of factors known to affect the general metabolism of the rat.

MATERIALS AND METHODS

Male albino rats of the Sprague-Dawley strain in groups of four to eight animals each were used throughout the study . The rats were placed in individual metabolic cages, and an accurate daily record of intake of food and water and excretion of feces and urine was kept. I^{131}-labeled sodium-*l*-thyroxine of high specific activity (Abbott Laboratories, Oak Ridge, Tennessee) was administered subcutaneously in doses not exceeding 1 μg. The I^{131} was measured by a well-type Geiger counter with a Tracerlab Scaler. All samples, including a pilot of the dose, were diluted to a constant volume. Radioactivity was expressed as a percentage of the dose. Excretion studies were carried out for 120 hours after injection of the labeled thyroxine.

Values for the fecal and urinary excretion of I^{131} after a physiologic dose of radioactive thyroxine were established for rats fed the "control" diet of ground "Friskies."[2] Four experimental diets were used as follows: 1. Soy-flour diet similar to that used by Van Middlesworth (30% soy flour,[3] 26% purified casein[4] and 10% cottonseed oil, plus a complete vitamin mixture). The iodine content of this mixture was 0.11 μg per gram; it is subsequently referred to as soy diet #1. 2. Soy diet #2, with the same formulation of soy diet #1 but containing 6 μg of iodine per gram. 3. Starch-casein diet, with the same formulation as soy diet #1 but with cornstarch substituted for soy flour. The iodine content of this diet was 0.07 μg/gm. 4. Low-iodine diet,[5] as formulated by Remington (4), containing 0.05 μg of iodine per gram.[5]

The excretion of I^{131} in bile after the use of labeled thyroxine was measured in rats fed the control diet and the two soy-flour diets. The bile was collected from the common bile duct through a polyethylene cannula. Fractionation by two-dimensional paper chromatography was carried out on the samples of bile and urine from rats feds soy diet #1 and soy diet #2.

The chromatograms were developed in butanol:dioxane:2N ammonium hydroxide overnight and then in tertiary amyl alcohol:2N ammonium hydroxide for 2 days.[6] Radioactive thyroxine and "radioactive" bile from donor rats were injected into the duodenum of rats fed the control diet and the soy-flour diets, and the I^{131} in urine and feces was determined.

Intestinal contents from rats fed the control diet and soy diet #2 were incubated for 24 hours with radioactive thyroxine and with radioactive bile, and aliquots of these were fractionated by precipitation with trichloroacetic acid and extraction with butanol.

The functional status of the thyroid was studied in rats fed the various diets by measurement of PBI, the 6-hour thyroidal uptake of I^{131} and the thyroidal weight, and by histologic examination.

RESULTS

The cumulative fecal and urinary excretion of I^{131} in various control groups of rats is shown in the accompanying figure. The mean values and

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[2] Albers Milling Company, Division of Carnation Co., Los Angeles, California.

[3] Cellu soy flour, Archer Daniels-Midland Co., Chicago, Illinois.

[4] Nutritional Biochemicals Co., Cleveland, Ohio.

[5] The iodine content of each diet was determined by our colleagues in the Section of Biochemistry.

[6] The authors are indebted to Dr. E. V. Flock, of the Section of Biochemistry, for performing the chromatographic studies.

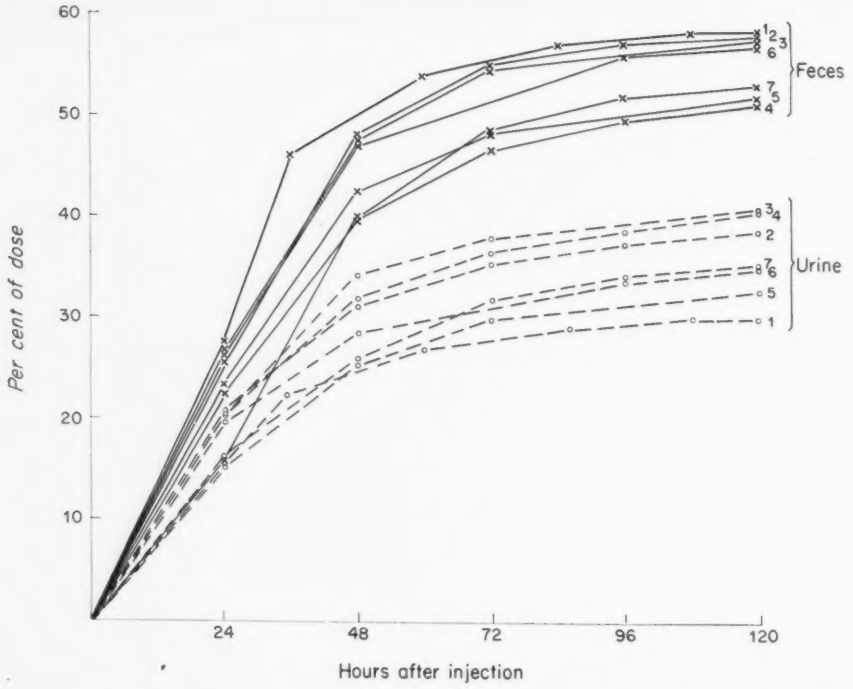

FIG. 1. Cumulative fecal and urinary excretion of I^{131} after tracer dose of radioactive thyroxine in seven control groups of rats. Each point is a mean value representing from four to 11 rats. The following data give the number of animals and the average weight for each control group: 1 = eight rats, 100 gm.; 2 = four rats, 150 gm.; 3 = four rats, 100 gm.; 4 = four rats, 150 gm.; 5 = 11 rats, 150 gm.; 6 = four rats, 190 gm.; 7 = four rats, 270 gm.

standard errors for the 39 control rats in the 5 days of study were 54.9 ± 1.3 percent of the dose in the feces and $34.5 \pm 0.8\%$ of the dose in the urine. The average daily weight of the fecal mass for a 150-gm. rat ingesting 15 gm. of food was 7 gm.

No difference from the control values for urinary and fecal excretion of I^{131} was found in groups of rats receiving injections of 500 μg of *l*-thyroxine, 12 μg of *l*-triiodothyronine or 2.5 mg. of cortisone acetate, when each drug was administered daily for 7 days prior to the tracer dose of labeled thyroxine and treatment was continued through the observation period. The feeding of potassium thiocyanate for 1 month, exposure of the rats to a temperature of 5° C for 1 week, the feeding of 40 mg. of neomycin sulfate prior to and during the study, and the administration of saccharo-1,4-lactone (an inhibitor of glucuronidase) failed to produce any significant difference in the proportional excretion of I^{131}.

Propylthiouracil (0.1% solution) given in the drinking water for 3 weeks before and during the study caused 64.0 ± 1.9% of the I^{131} to appear in the feces, while 26.9 ± 0.5% of the dose was in the urine. No significant difference was evident in the weight of the feces compared to control values.

The daily injection of 0.1 mg. of epinephrine for 7 days prior to and during the study resulted in a fecal excretion of 65.1 ± 0.5% of the I^{131} injected and a urinary excretion of 34.1 ± 0.5% of the dose without any alteration in the fecal weight or in the frequency of the fecal ejections. Administration of 2,4 dinitrophenol in an oral dose of 10 mg. per day for 2 weeks caused 70.3 ± 1.3% of the injected I^{131} to appear in the feces, while 30.0 ± 1.5% appeared in the urine.

TABLE 1. EXCRETION OF I^{131} OF RATS FED VARIOUS DIETS

Diet	Excretion of I^{131}*		Feces excreted per 24 hours, gm.	Total 24-hour radioactivity in bile, per cent of dose†
	Feces	Urine		
Remington	(8) 30.8 ± 1.3	45.6 ± 2.6	1.0	—
Starch-casein	(4) 19.4 ± 1.5	44.3 ± 2.8	1.0	—
Soy diet #1	(8) 27.8 ± 0.9	42.8 ± 1.3	3.5	(4) 23
Soy diet #2	(20) 27.4 ± 0.7	56.5 ± 1.1	3.5	(4) 25
Control	(39) 54.9 ± 1.3	34.5 ± 0.8	7.0	(4) 23

* Cumulative excretion (5 days) of I^{131} expressed as per cent of radioactivity injected (mean ± S.E.). Numbers in parentheses indicate number of rats per group.

† Bile collected via cannula for 24 hours after injection of radioactive thyroxine.

The fecal excretion of I^{131} was increased to 67.0 ± 3.1% of the amount injected in rats given 2 gm. of psyllium hydrophilic mucilloid (metamucil) in their food and 4 ml. of an emulsion of mineral oil and phenolphthalein (agoral) in their drinking water per day. The urine contained 31.1 ± 2.0% of the I^{131} injected.

A decrease in fecal radioactivity to 35.8 ± 2.0% of that injected was accomplished by limiting the intake of food so that the weight of the fecal mass was only one half of that observed in a group of rats given an unlimited quantity of food. The urine contained 42.3 ± 1.4% of the radioactivity by the end of the 5-day period.

Table 1 shows the comparative values for excretion of I^{131} by groups of rats fed the experimental diets. The control group had the greatest excretion of I^{131}, namely 54.9% and the highest fecal weight per 24 hours. The fecal excretion of I^{131} in the two groups fed the soy-flour diets was one half as much as that of the control group. The weight of feces excreted in 24 hours was also one half of that in the control group. The rats fed the iodine-deficient soy diet #1 excreted less I^{131} in the urine (42.8%) than did those fed soy diet #2 (56.5%); this is a difference of 14 percentage points. The biliary excretion of I^{131} was the same in these three groups. The rats fed the starch-casein diet had a fecal excretion of I^{131} that was about one third as

much as that of the control group and approximately two thirds of that of the rats fed soy flour. The stool weight was greatly reduced in the starch-casein group compared to that in the soy-flour and control groups. The excretion of I^{131} by rats on the Remington diet was similar to that by rats eating the soy diet #1.

Chromatographic fractionation of bile from the control rats and from those fed the soy diet #2 did not demonstrate any difference in the types of radioactive substances present in the specimens. Paper chromatography of urine from rats fed the soy diet #2 revealed that 91% of the radioactivity was in the form of inorganic iodide.

When radioactive thyroxine was instilled directly into the duodenum of four rats fed the soy diet #2, the mean fecal excretion in 48 hours represented 28.1% of the radioactivity, while 30.7% appeared in the urine. This contrasted with a fecal excretion of 54% and a urinary excretion of 27% when radioactive thyroxine was instilled into the duodenum of rats receiving the control diet. When aliquots of "radioactive" bile (from the bile ducts of rats given 50 micrograms of radioactive thyroxine) were instilled into the duodenum of four rats fed the control diet and four rats fed the soy diet #2, the mean 48-hour fecal excretion of radioactivity was 61.6% for the control rats and 31.8% for the latter group.

Incubation of radioactive thyroxine or radioactive bile with the intestinal contents of rats fed the soy diet #2 did not produce any difference in the proportional amounts that were precipitable by trichloroacetic acid or soluble in butanol when compared to incubation with the intestinal contents from rats receiving the control diet.

The soy diet that was deficient in iodine caused a decrease in PBI from a control value of 5.5 μg per 100 ml. to 1.8 μg but surprisingly did not produce a great increase in the thyroidal uptake of I^{131}. The thyroidal weight of this group of rats was increased only slightly from the control level of 5.5 mg. per 100 gm. of body weight. The thyroid showed some increase in weight after the rats were fed the soy diet #2 for 31 days. The uptake of I^{131} in the rats receiving the diet with abundant iodine was less than that of the control group despite a decreased PBI. The histologic picture of the thyroid in the rats fed the soy diet #2 suggested less cellular activity as compared to that in the rats fed soy diet #1. The Remington diet caused a pronounced increase in the uptake of I^{131} and an increase in thyroidal weight. These aspects of thyroidal function are summarized in Table 2.

COMMENT

Our observations on the fecal excretion of I^{131} in rats fed the starch-casein diet and the soy diet #1 are similar to the findings of Van Middlesworth (1). However, Beck (2) reported fecal excretion of more than 100% of the injected dose of radioactive thyroxine in soy-fed rats, while the control rats fed starch-casein excreted 70% of the dose after 90 hours. Th

addition of iodine to soy diet #1 did not change the fecal weight or the proportion of radioactivity excreted in the feces but did allow a greater recovery of I^{131} in the urine, showing that the I^{131} not excreted in the feces actually is re-entering the blood stream.

The soy-flour diet allows for greater reabsorption of thyroxine from the gastrointestinal tract than does the control diet. This statement appears to be justified since the rate of excretion of radioactivity in the bile and the relative concentration of I^{131} compounds in the bile are the same in both diets and since no difference exists in the transformation of thyroxine or products of biliary excretion brought about by the intestinal contents in

TABLE 2. THYROIDAL FUNCTION IN RATS FED VARIOUS DIETS

Diet	Number of days on diet	Iodine content of diet, μg./gm.	PBI, μg/100 ml.*	Thyroidal I^{131} uptake at 6 hours, per cent of dose*	Thyroidal weight, mg./100 gm. of body weight*	Histology
Control	30	6.0†	(5) 5.5±0.4	(38) 1.47±0.1	(38) 5.5±0.1	Minimal colloid; tall columnar cells
Soy diet #1	17	0.11	(4) 1.8±0.3	(8) 1.82±0.1	(8) 5.9±0.4	Colloid more abundant; medium cell height
Soy diet #2	17	6.0‡	—	(8) 0.34±0.1	(8) 5.5±0.1	Abundant colloid; flattened cells
	31	6.0‡	(5) 3.6±0.1	(12) 0.62±0.1	(12) 6.1±0.3	
Remington	47	0.05	—	(10) 3.97±0.2	(10) 7.4±0.3	—

* Numbers in parentheses indicate number of observations. Mean values and standard errors are given.
† Total iodine content; 1.1 μg of this was organic iodine.
‡ Iodine added to the diet in the form of potassium iodide.

both diets. Beck (2) did not find any quantitative or qualitative difference in the biliary excretion of radioactivity in rats fed an iodine-deficient soy-flour diet or a starch-casein diet. The combined urinary and fecal content of I^{131} after 5 days was the same in both groups, and there was no difference in the type of radioactive compounds found in the feces. The only measurable factor to account for the difference in fecal excretion in our study is the difference in the weight of the fecal mass expelled per day.

The actual intake of iodine by the rats in Van Middlesworth's study was not indicated. In our study, a 100-gm. rat eating the soy diet without added potassium iodide received a maximum of 1.4 μg of iodine per day. It has been estimated that between 1 and 2 μg of iodine per day is the daily requirement for the prevention of goiter in rats (5). The low iodine content of the soy diet can account for the high thyroidal uptake of I^{131} found by

Van Middlesworth. The production of a goiter weighing 14 mg. per 100 gm. of body weight in Van Middlesworth's investigation could be the result of iodine deficiency; however, the appearance of a goiter in rats after just 2 weeks of iodine deficiency is unusual. Money and associates (6) found that 106 days of an iodine-deficient diet was necessary in rats to produce a goiter of 9.4 mg. per 100 gm. of body weight.

Although McCarrison (7) and also Sharpless and co-workers (8) reported the occurrence of large goiters in rats fed soy flour despite an intake of large amounts of iodine, it was shown later by Wilgus and associates (9) and Halverson's group (10) that the goitrogenic property of soy flour was completely counteracted by the addition of an adequate amount of iodine. The observation in our study of a slight increase in thyroidal weight after feeding the soy diet #2 for 5 weeks (the P value for the difference is 0.02) suggests that a goitrogenic factor may be present in soy flour but the gland is not hyperactive nor does it take up I^{131} very avidly. The existence of "soybean goiter" cannot be denied unequivocally on the basis of this study, but it appears that the mechanism is not likely one of excessive loss of thyroxine in the feces.

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NOTES AND COMMENTS

RABBIT PREGNANCIES AFTER IMMUNIZATION WITH EXTRACT OF THYROID GLAND[1]

ABSTRACT

A rough correlation was observed in mother rabbits immunized to an extract of rabbit thyroid, between the height of antithyroid antibody titer achieved and the incidence and degree of histologic changes of "thyroiditis." No significant abnormality was observed in the thyroids of mother rabbits given saline or immunizing injections to hog or human thyroid gland. Although transplacental transfer of thyroid antibody was demonstrated and there was a high incidence of fetal death in rabbit-thyroid immunized rabbits, no evidence of congenital hypothyroidism was demonstrable in the fetuses.

INTRODUCTION

Morphologic changes similar to those in Hashimoto's struma have been produced in the thyroid glands of rabbits by active immunization with rabbit-thyroid extracts (1, 2). Roitt *et al.* (3) demonstrated thyroid auto-antibodies in the sera of patients with Hashimoto's disease. Doniach and Roitt (4) found thyroglobulin precipitins in the sera of 6 patients with "spontaneous" non-goitrous myxedema. These considerations led us to investigate the possibility that thyroid auto-antibody in the mother might be transmitted through the placenta to combine with the developing fetal thyroid gland and produce intra-uterine hypothyroidism (5, 6). This communication is a report of the study in rabbits.

METHODS

Fifty female New Zealand White and Sandy Flemish rabbits weighing 8½ to 13 pounds were divided into four groups. Twenty-three rabbits comprising Group I were "immunized" with a saline extract of rabbit-thyroid with the complete Freund's adjuvant at 4 week intervals. Eight (Group II) were similarly "immunized" against hog thyroid extract and 8 against human thyroid extract (Group III). Eleven were similarly injected with either saline only, or were given no injections (Group IV).

All rabbits were bled at the second and third week after the "immunizing" or saline injection and were mated after the first to fifth injection. A caesarean section was performed approximately two days before the expected date of delivery, and 24 hours after a tracer dose of 25 microcuries of I^{131} was given subcutaneously to the mother. Blood was drawn from each fetus for a serum protein-bound iodine, PBI, (7), and when possible, a butanol extractable iodine, BEI, determination was done (8).[2] The fetal and maternal thyroid glands were blotted, weighed, and counted in a scintillation well counter with the aid of a spectrometer, then fixed, sectioned, and stained. In 7 mother rabbits after caesarean section, the abdomen was closed and counts were made over the thyroid gland before and after the administration of 0.3 gm. of potassium thiocyanate, KSCN, intraperitoneally (9). Thyroid antigen was prepared and administered by the method of Witebsky and Rose (10) from rabbit, hog, or human thyroid glands obtained by surgical thyroidectomy. Witebsky's modification of Boyden's tanned red blood cell hemagglutination technique (10) was used initially to demonstrate the presence or absence of circulating thyroid autoantibodies.

Pathology

From two to six microscopic sections of each thyroid gland examined were graded without knowledge of immunologic data. Histologic grades were assigned according to the type and extent of the inflammatory reaction and amount of altered colloid (i.e. granularity). Final grading included: negative (0) and positive (grades 1, 2, 3 and 4) according to the extent and types of specific histologic changes.

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[2] Bio-Science Laboratories, 12330 Santa Monica Boulevard, Los Angeles 25, California.

TABLE 1

GROUP I (Rabbit-thryoid Immunized)

a. Maximum Maternal Titer <500

Rabbit number	Number of injections	Time from first injection to sacrifice (months)	Max. titer	Titer at delivery		Maternal serum			Fetal pooled serum			Maternal I^{131} thyroid uptake net cpm. at 24 hr.	Litter I^{131} thyroid uptake net cpm. at 24 hr.	Mean fetus I^{131} thyroid uptake net cpm. at 24 hr.	Litter size (number of placentas)	Number of dead fetuses	Histologic grade of thyroid
				Mat	Fet	PBI	BEI	I_2	PBI	BEI	I_2						
						(μg %)			(μg %)								
149	6	6	+12	+3	neg	0.9	0.8	<0.5	0.5	0.4	0.5–1.0	432,196	962,815	120,352	8	0	0
157	4	4½	+48	+3	+3	1.4	0.8	0.2–0.5	1.5		0.2–0.5	596,395	1,027,668	256,917	4	0	0
159	4	4½	+6	neg	+3	1.3	0.7	<0.2	1.3	0.5	<0.2	233,652	981,238	70,088	15	1	0
155	4	4½	+6	+3	+3	0.7	0.8	<0.2	0.8	0.8	<0.3	461,375	1,652,475	156,248	10	0	0
158	4	4½	+6	+3	neg	1.6	1.4	<0.2	0.8	0.8	<0.4	625,345	805,886	89,549	12	3	0
161	4	4½	+3	+3	neg	1.8	1.0	<0.5	1.4	0.6	<1	591,800	737,062	137,412	6	1	1
162	4	5	+3	neg	+3	1.8	0.7	<0.5	1.8	1.5	<0.5	118,546	1,707,097	179,677	10	0	0
216*	5	5	+80	neg	neg	1.5		>25				1,048,904	332,580	146,290	7	5	0
218	5	5	+5	neg	neg				1.0	0.6	<0.6	567,977	824,840	91,649	8	6	0
224	5	5½	+20	neg	neg	1.8		<0.5	1.5		0.3–0.8	493,159	631,336	126,267	6	1	0
				Mean		1.4	0.9	~3	1.2	0.7	0.7						
				S.D.±		0.4	0.3		0.4	0.4							

b. Maximum Maternal Titer 500–1,000

148	6	6	+768	+24	+12	1.8		<0.5	1.4	1.4	0.5–1.0	392,396	1,473,287	147,329	10	0	1
164	4	4½	+768			1.4	1.3	<2	1.1	0.2	<2	442,793	811,987	135,331	6	0	0
201*	5	5½	+768			1.4	1.2	<1.0	0.9	0.6	1.5	976,124	651,737	65,174	10	0	1
175	6	7	+768	+10	+5	0.9		<2.0				766,700	488,776	61,097	13	4	0
				Mean		1.4	1.3	<1.4	1.1	0.7	<1.5						
				S.D.±		0.4			0.3	0.6							

c. Maximum Maternal Titer >1,000

151	6	6	+1,536	+348	+48	1.1	0.9	<0.5	0.9	0.8	<0.5	821,615	994,161	198,832	5	0	3
156	4	4½	+1,536	+3	+3	1.0	0.8	<2	1.2	0.9	<2	677,295	1,018,387	127,298	8	0	2
198*	4	3	+1,536	+320	+320	2.0	1.6	<25–500	1.3	1.1	0.5–1.0	1,647,702	661,958	94,565	9	2	1
192*	3	4	+1,536	+640	+640	1.6	1.1	0.5	2.4		7–8	355,819	676,190	169,048	4	0	2
223*	4	5	+5,120	+1,280	+2,560							697,672	948,411	118,551	9	1	3
215	4	4½	+2,560	+5	+10							367,911	648,858	81,107	13	5	2
225	4	4½	+3,560	+40	+20	1.0		<1				479,697	1,380,748	276,150	8	3	0
166*	7	8½	+1,536	neg	+20	0.5	0.4	0.5				607,450	772,915	128,819	12	6	4
214*	5	5	+1,280	+40	+40	1.5	1.1	>25				1,118,984	343,799	171,900	7	5	4
				Mean		1.2	1.0	<150	1.5	0.9	<3						
				S.D.±		0.5	0.4		0.7	0.2							

* These rabbits had KSCN tests. No discharge of thyroidal I^{131} in any instance.

(Continued on next page)

TABLE I—(*Continued*)

Rabbit number	Number of injections	Time from first injection to sacrifice (months)	Max. titer	Titer at delivery		Maternal serum			Fetal pooled serum			Maternal I^{131} thyroid uptake net cpm. at 24 hr.	Litter I^{131} thyroid uptake net cpm. at 24 hr.	Mean fetus I^{131} thyroid uptake net cpm. at 24 hr.	Litter size (number of placentas)	Number of dead fetuses	Histologic grade of thyroid
				Mat	Fet	PBI	BEI	I_2	PBI	BEI	I_2						
						(μg %)			(μg %)								
Group II (Hog-Thyroid Immunized)																	
70	1	2		12,288	1,536	3.4	1.8	<10	2.6	1.7	<10	95,973	185,646	13,260	15	1	0
87	1	1½		24,576	24,576							241,531	437,918	109,480	4	0	Lost
86	1	2		49,152		1.4		<10	1.1		<10	522,193	875,219	109,402	8	0	0
102	1	2		384	384	1.9	1.5	<10	1.8	1.0	<10	86,507	332,641	41,580	11	1	0
105	1	3		68	68	2.0	0.6	<10	1.3	1.0	<10	72,911	660,667	60,061	12	1	0
101	1	3½		12,288	3,072	1.6	1.6	<10	1.2	0.7	<10	240,244	287,595	71,889	6	2	0
104	1	4		3,072	768							172,180	696,699	116,117	7	1	Lost
119	1	2		1.5 mil	1.5 mil							99,967	472,849	67,550	8	1	Lost
				Mean		2.1	1.4	<10	1.6	1.1	<10						
				S.D.		0.8	0.5		0.6	0.4							
Group III (Human-Thyroid Immunized)																	
91	1	2		245,760	15,360	1.1	1.3	<10	2.5	2.2	<10	585,044	1,164,661	129,407	9	1	0
96	1	2				1.9	1.7	<10	1.5		<10	273,190	382,778	95,695	8	4	0
106	1	2½		786,432	24,576	2.8	2.1	<10	1.7	0.5	<10	142,284	150,089	15,009	10	0	0
116	1	2		786,432	196,608	1.5	0.8	<10	0.9	0.4	<10	138,723	328,619	65,724	6	1	0
117	1	2		1.5 mil	12,288				1.1	1.1	<10	181,614	1,523,312	152,341	10	0	0
114	1	2		49,152	24,576	2.1	0.7	<10	1.5	0.7	<10	159,427	545,221	54,522	10	0	0
115	1	2		1.5 mil	1.5 mil							137,737	563,496	70,437	8	0	Lost
118	1	3		1.5 mil	1.5 mil							83,810	567,037	63,004	9	0	Lost
				Mean		1.9	1.3	<10	1.5	1.0	<10						
				S.D.±		0.6	0.6		0.6	0.7							
Group IV† (Saline Injected, or Non-Injected, Controls)																	
15	7	8				2.1		<10				75,304	137,050	22,842	9	0	0
52	6	8				1.7		<10				73,433	222,752	44,550	7	0	0
54	7	9				2.1		<10	1.2			391,822	305,923	43,703	9	2	0
126	0					2.3	2.2	<10	1.9	1.7	<10	142,561	63,818	21,273	3	0	0
127	0					0.7	0	<10	0.4	0	<10	188,472	1,406,306	140,631	10	0	0
130	0					1.2	1.0	<10	0.2	0	<10	85,421	375,331	41,703	9	0	0
131	0					1.0	0.7	<10	4.0	1.8	<10	215,439	497,488	45,226	11	0	0
128	0											152,494	681,825	75,758	9	0	0
207	0					0.8	0.5	<2	1.3	0.6	<2	117,046	855,723	106,965	8	0	0
208	0					0.9	0.5	2	0.6	0.2	0.5–1.0	446,000	862,393	95,821	9	0	0
211	0					1.5	1.1	0.5	1.5	1.0	0.5	1,366,418	474,668	59,334	8	0	0
				Mean		1.43	0.86	8	1.39	0.76	6						
				S.D.±		0.59	0.70		1.2	0.77							

† These rabbits gave negative titers against hog, human and rabbit thyroid antigens.

RESULTS

All data are presented in Table 1 for the four groups of rabbits as outlined under "Methods."

No significant abnormality was observed histologically in the thyroid glands of saline injected or non-injected rabbits or in rabbits "immunized" against hog and human thyroid glands to relatively high titers. A rough correlation was observed in the rabbit-thyroid immunized mother rabbits between the height of antithyroid antibody titer achieved and the incidence and degree of histologic changes of "thyroiditis." None of the fetal thyroid glands in any of these groups showed changes of the type seen in the thyroid glands of mother rabbits of Group I.

Transplacental transfer of thyroid antibody was demonstrated in every hog- and human-thyroid immunized, and in all but 3 rabbit-thyroid immunized rabbits, in which antibody titers were obtained at delivery.

A fairly high percentage of litters was found to have one or more dead fetuses at caesarean section. The incidence of affected litters was highest in the rabbit-thyroid and hog-thyroid "immunized" groups. The percent of affected fetuses in each affected litter was highest in the rabbit-thyroid "immunized" group. No significant difference between the various groups was observed in brain size or configuration other than that associated with fetal death.

Although the intra-litter incidence of dead fetuses was highest in rabbits with demonstrable antibody titers to rabbit-thyroid antigen, there was no significant difference within the rabbit-thyroid immunized groups or between Groups I, II, III and IV with regard to maternal and fetal serum PBI and BEI concentrations or 24-hour thyroidal I^{131} "uptake" determinations.

KSCN testing *in vivo* failed to demonstrate impaired organic binding of iodine in any of the 7 rabbit-thyroid immunized rabbits in spite of the fact that rabbits #166 and #214 had grade 4 histologic changes.

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ANTI-ENDOTOXIC ACTIVITY OF CORTICOSTEROIDS

ABSTRACT

A method for assaying corticosteroid activity based on the protection against the lethal action of bacterial endotoxin has been developed.

Linear relationships between survival and dose of endotoxin were observed when a mixture of endotoxin and steroid were given intracardially to adrenalectomized rats.

The relative activities of corticosteroids in protecting against endotoxins are different from their apparent anti-inflammatory activities.

The protective action of cortisone and cortisol against the lethal effect of bacterial endotoxin has been demonstrated in rabbits, rats, mice, and chick embryos (1). Lewis and Page (2) suggested that the protective effect of corticosteroids against the lethal action of typhoid vaccine in adrenalectomized rats provided a basis for a steroid assay. Halberg *et al.* (3) demonstrated that when adrenalectomized mice were challenged with Brucella endotoxin not only cortisone and cortisol but also 9-a-fluorohydrocortisone, and, to some degree, aldosterone, prevented hypothermia in the mice and prolonged their survival. Levitin, Kendrick, and Kass (4) found that the protective action of corticosteroids could be measured in intact animals, but that unless the steroids were given directly into the circulation their effect might be obscured by differences in the rates with which the steroids were mobilized from local tissue sites of deposition. These workers observed that in rats corticosterone was almost as efficacious as cortisol when given intracardially with endotoxin, whereas these two steroids differed in their protective action after subcutaneous administration.

We, therefore, devised an assay in which adrenalectomized rats were injected intracardially with mixtures of corticosteroids and endotoxin. While these studies were in progress (5), Gass and Umberger (6) reported a suggested assay in which adrenalectomized rats were given large doses of endotoxin intraperitoneally, and the action of cortisol in prolonging survival and in diminishing hypothermia was determined; no information concerning other steroids was presented.

Female white rats weighing 100–150 grams were adrenalectomized under light ether anesthesia 24 hours before use. The animals were given saline drinking water. Endotoxin and steroid were given intracardially in a single mixture, unless otherwise noted. Although the end-point was taken as death after 24 hours, most animals succumbed within the first 5 hours after injection. Animals dying during the first hour after injection were considered to have died of trauma incident to the injection or to the embolic action of undissolved steroid, and were removed from the experiment. Pericardial hemorrhage occurred in fewer than 1% of the animals injected by the intracardiac route. The dose of endotoxin given to each animal was one which caused approximately 90% of test animals to die within 24 hours, under the conditions cited. Environmental temperature of the animal rooms was controlled at 65–70° F. The manner of injection, the preparation of steroids for injection, and the preparation of the *S. typhi* endotoxin have been described previously (4).

RESULTS AND DISCUSSION

The protective effect of cortisol in this system is shown in Figure 1. In this, as in subsequent experiments, the percentage of dead animals receiving endotoxin and steroid

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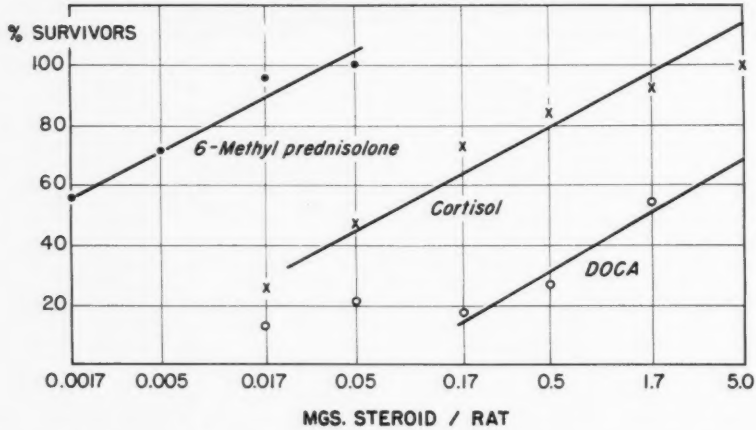

FIG. 1. Anti-endotoxic activity of corticosteroids in adrenalectomized rats. Each dose of corticosteroid was given intracardially with an LD_{90} dose of endotoxin.

was subtracted from the percentage of dead animals receiving endotoxin alone in the same experiment. The protective action is more or less a linear function of the logarithm of the dose of cortisol per rat within at least a hundred-fold range of concentration of steroid. The relative effects of desoxycorticosterone acetate and of 6α-methyl prednisolone are also shown and illustrate the general linearity of protection over a wide range of steroid concentration. The dose of steroid which would protect 50% of animals (PD_{50}) was calculated. The PD_{50} values of a variety of steroids are given in Table 1.

The different methods of assay of anti-inflammatory activity of corticosteroids do not agree closely (7, 8). Thus, it is not surprising that the anti-endotoxic action of several of the steroids tested does not parallel closely their apparent anti-inflammatory activity

TABLE 1. PROTECTIVE EFFECT OF CORTICOSTEROIDS ON ADRENALECTOMIZED RATS GIVEN ENDOTOXIN

Compound	No. of animals used in assay*	PD_{50} mgs./rat†	PD_{50} relative to cortisol
Cortisol	271	0.06	1.0
Cortisol acetate	161	0.05	1.2
11-deoxycortisol	53‡	0.06	1.0
Corticosterone	194	0.15	0.4
11-dehydrocorticosterone	99	0.17	0.35
11-deoxycorticosterone acetate	276	>1.7	<0.04
Progesterone	59‡	1.7	0.04
11-OH progesterone	78	0.3	0.2
17 β-OH progesterone	54‡	>0.5	<0.12
Prednisone	37‡	0.03	2.0
Prednisolone	40	0.03	2.0
6 α-methyl prednisolone	107	<0.0017	>35.0

* This does not include control animals.

† The percentage of dead animals receiving both endotoxin and steroid was subtracted from the percentage of dead animals receiving endotoxin alone in the same experiment. Extrapolation from the plot of percentage survivors versus log dose of steroid gave the PD_{50} (see Figure 1).

‡ These compounds were so insoluble that animals given higher concentrations died within 30 minutes of injection, probably due to emboli. Such animals were not included in the PD_{50} calculation.

as measured experimentally, and clinically (9). Nevertheless, the activity of 6α-methyl prednisolone is noteworthy since it is generally agreed that this substance is 4–5 times as active as cortisol as an anti-inflammatory agent, and the present data indicate that it is more than 35 times as active as cortisol as an anti-endotoxic agent—a difference that could hardly be explained on the basis of variation in the methods used.

SUMMARY

The anti-endotoxic action of corticosteroids provides the basis for an assay in which adrenalectomized rats are given intracardially a mixture of endotoxin and corticosteroid. The anti-endotoxic action of several corticosteroids has been found to differ from their apparent anti-inflammatory activity.

Prednisone and prednisolone are twice as active as cortisol as antiendotoxic agents and 6α-methyl prednisolone is over 35 times as active as cortisol.

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BIOGENESIS OF ESTROGENS BY THE HUMAN OVARY:[1] THE CONVERSION OF ANDROSTENEDIONE-4-C^{14} TO ESTRONE AND ESTRADIOL[2] IN HIGH YIELD

ABSTRACT

Androstenedione-4-C^{14} was incubated with tissue from the polycystic ovaries of a woman treated preoperatively with gonadotropic hormones. Radioactive estrone and estradiol were isolated. The total amount formed approximated 15% of the starting material. The possible significance of such a relatively high yield is discussed.

It has been demonstrated that human ovarian tissue converts testosterone to estrogens (1, 2), but in such low yield as to raise some question concerning the importance

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[2] This is paper No. 5 in a series devoted to biogenesis of the estrogens by the human ovary.

of this reaction in ovarian estrogen biosynthesis *in vivo*. The remarkable capacity of placental tissue for converting androgens to estrogens (3) lends strong support to the thesis that androgens are involved in the placental biosynthesis of the high levels of estrogen in pregnancy. In a current series of studies in this laboratory the qualitative and quantitative aspects of ovarian estrogen biosynthesis are being investigated. The tissue preparations used are from greatly enlarged, polycystic ovaries from women treated pre-operatively with pituitary gonadotropic hormones. Such ovaries elaborate estrogen in unusually large amounts as indicated by the finding of levels of urinary estrogens during injections that are normally encountered only in early pregnancy (4, 5). If androgens are of major importance in the ovarian biosynthesis of estrogens, such hyperactive ovaries should show an increased capacity for androgen to estrogen conversion.

METHODS AND MATERIALS

The ovaries for the present study were obtained at surgery from a normally menstruating woman who had received 50 mg. equivalents of Armour Standard F.S.H. daily for 19 days, plus 20,000 I.U. of HCG during the last 4 days before operation.[3] Greatly enlarged polycystic ovaries were obtained, and urinary estrogens on the 13th to 15th days of treatment ranged from 350 to 450 μg per 24 hours.[4] The follicular cyst linings (11.2 gm.) were homogenized and centrifuged for 20 minutes at 800×g. The supernatant fluid was incubated for 3 hours with 1.46×10^6 c.p.m. of androst-4-ene-3,17-dione-4-C^{14} that had been previously purified on a Celite column.[5] The methods used for tissue preparation and incubation, extraction of the tissue for steroids, fractionation for phenolic metabolites, measurement of radioactivity and of estrogens, Celite column chromatography, further purification and establishment of identity with carriers have been previously described (7, 8, 9).

RESULTS

The incubation mixture was extracted 3 times with 6 volumes of chloroform and the residue (tissue and aqueous phase) treated with 20 volumes of hot acetone. After filtration the acetone-aqueous mixture was evaporated to 5 ml. and extracted 3 times with 30 ml. chloroform. The combined chloroform extracts contained 1.20×10^6 c.p.m. or 82% of the starting radioactivity. This was partitioned between pentane and 90% methanol and the methanol soluble material separated into "estriol," "estrone-estradiol" and "neutral" fractions as previously described (7). Total recovery was 1.18×10^6 c.p.m.; with 3.6% in the water soluble "estriol" fraction, 24.4% in the NaOH soluble "estrone-estradiol" fraction and 72.0% in the residual benzene:petroleum ether or "neutral" fraction. The neutral steroids were set aside for later study.

The "estriol" fraction (41,900 c.p.m.) was run on a Celite column with 200 μg each of estradiol-17β, 16-*epi*estriol and estriol added as carriers. Four distinct peaks of radioactivity were observed which did not coincide with the peaks for any of the carriers and were not identified. There was no concentration of counts in the region of the carrier estriol section.

The "estrone-estradiol" fraction (287,500 c.p.m.) was run on a Celite column with

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[3] The pituitary follicle-stimulating hormone (ovine) was obtained from the Armour Pharmaceutical Co., and the human chorionic gonadotropin (Follutein) from E. R. Squibb and Sons through the courtesy of Dr. Edward F. Reifenstein. The authors are indebted to Dr. William P. Mulligan for assistance in obtaining the gonadotropin-stimulated ovaries.

[4] Urinary estrogens (estrone, estradiol and estriol) were measured by the modification of the J. B. Brown method developed in this laboratory (6). Total estrogen output in this patient was about 10 times as high as that found in normally menstruating women at the ovulatory peak of estrogen excretion (6).

[5] The androstenedione-4-C^{14} for this experiment was obtained from the New England Nuclear Corp. (7.576 microcuries per mg.).

TABLE 1. PURIFICATION OF RADIOACTIVE ESTRONE FROM ANDROSTENEDIONE-4-C^{14}

Purification steps (successive)	c.p.m.	μg	Specific activity
Celite column (18 tubes)	181,600		987 ± 33*
Pool assay	179,100	186	963
Saponification and alkali wash	154,400	179	863
Methylation and NaOH: petroleum ether partition	148,100	176	841
Alumina chromatography	141,800	168	844

* Ave. ±S.D. of 18 tubes that made up the pool.

200 μg each of androstenedione, estrone and estradiol-17β added as carriers. Sixty-seven per cent of the radioactivity was recovered in the estrone section and 26.3% in the estradiol section. The only other concentration of radioactivity was in the androstenedione section where 2% of the total c.p.m. applied to the column was recovered.

Purification of Radioactive Estrone. Table 1. Statistical analysis of the estrone peak from the column revealed a constant specific activity over 18 tubes of 987 ± 33 c.p.m./μg with a coefficient of variation of 3.3% . These 18 tubes were pooled and assayed, purified by saponification and alkali wash, methylated and subjected to further purification by hydrogen peroxide treatment followed by NaOH:petroleum ether partition. The petroleum ether extract was then run on an alumina column. At each step the bulk of the radioactivity ran with the estrone carrier. There was some loss of radioactivity with saponification, but the specific activities thereafter were constant within the range of error of the methods of assay. The total radioactivity of the 18 tubes from the Celite column corrected on the basis of the change in specific activity and the losses with subsequent purification, was estimated to represent 158,900 c.p.m. of radioactive estrone, or 10.7% of the androstenedione-4-C^{14} added to the tissue for incubation.

Purification of Radioactive Estradiol-17β. Table 2. In the estradiol section from the column, fluorescence assays were interfered with by quenching except in the peak tube, the specific activity of which agreed with that of the pool of 5 tubes taken for purification. The pool was first run on a chloroform:formamide paper chromatogram and the eluted estradiol section purified by saponification and alkali wash before being assayed for fluorescence and radioactivity. This was followed by methylation, hydrogen peroxide purification, NaOH:petroleum ether partition and column chromatography on alumina. At each step the bulk of the radioactivity accompanied the estradiol carrier, and the specific activity was constant after methylation. The total radioactivity of the 5 tubes from the Celite column, corrected on the basis of the slight drop in specific activity and the losses with subsequent purification were estimated to represent 64,800 c.p.m. of

TABLE 2. PURIFICATION OF RADIOACTIVE ESTRADIOL 17β FROM ANDROSTENEDIONE-4-C^{14}

Purification steps (successive)	c.p.m.	μg	Specific activity
Celite column (5 tubes)	69,600		
Peak tube	25,700	69.0	373
Pool assay	61,000	169	361
Paper chromatography Chloroformformamide	46,600	132	353
Methylation and NaOH: petroleum ether partition	41,000	123	334
Alumina chromatography	38,300	112	342

radioactive estradiol-17β, amounting to 4.4% of the androstenedione-4-C^{14} starting material.

DISCUSSION

An estimated 15% of the androstenedione-4-C^{14} substrate, therefore, was converted to estrogens that could be identified as estrone and estradiol. This is a less striking yield than the 50 to 60% from experiments with placental tissue (3) but considerably greater than the 0.1 to 0.2% conversion of androgens to estrogens indicated by studies with human ovaries by previous investigators (1, 2). Such a high yield, obtained from ovaries known to be hyperactive *in vivo* in the elaboration of estrogen, lends support to the thesis that androgen to estrogen conversion is of physiological importance in the biogenesis of estrogens by the human ovary.

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THE EFFECT OF CORTISONE UPON AMINO ACID ACTIVATING ENZYMES OF RAT LIVER CORRELATED WITH INCREASED TRYPTOPHAN PYRROLASE ACTIVITY[1]

ABSTRACT

Studies were conducted concerning possible variations in the activity of amino acid activating enzymes accompanying increased tryptophan pyrrolase activity in rat liver resulting from cortisone injection. Though cortisone injection produced the expected significant increase in tryptophan pyrrolase activity, it caused no significant rise in either the total or specific activity of several amino acid activating enzymes.

It would appear that the administration of cortisone results in the increased synthesis of certain liver protein fractions (1–4). There is increasing indirect evidence that enhanced tryptophan pyrrolase activity following cortisone administration may involve the *de novo* synthesis of the apoenzyme. Ethionine has been shown to inhibit the cortisone-evoked stimulation of the enzyme and the inhibition is reversed by methionine. (5).

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[1] This work was supported by U. S. Public Health Service Grants RG-5475 and A-1469

TABLE 1. EFFECT OF CORTISONE INJECTION UPON THE ACTIVITY OF RAT LIVER AMINO ACID ACTIVIATING ENZYMES AND TRYPTOPHAN PYRROLASE

Values given as percent of controls

Expt.	P-32 Exchange					Tryptophan pyrrolase activity
	No added amino acid	Amino acid-dependent exchange				
		Leucine	Valine	Methionine	Tryptophan	
1	72	67	66	76	82	—
2	70	66	95	105	98	433
3	80	87	91	53	101	—
4	104	129	146	168	—	269
5	91	99	106	128	—	526
6	127	105	89	72	—	320
Average	91	92	99	100	94	387

Feigelson (6) has reported an accumulation of large amounts of apoenzyme in the cell sap following cortisone administration. If cortisone stimulates the *de novo* synthesis of the apoenzyme of tryptophan pyrrolase, it would be of interest to test the possible influence of cortisone upon the first step of protein synthesis, amino acid activation. Both estrogens and androgens have been reported to stimulate the activity of amino acid activating enzymes upon *in vivo* administration (7, 8). In an attempt to shed further light on this question and to study certain aspects of the hormonal control of protein synthesis, the effects of cortisone upon the activity of amino acid activating enzymes of rat liver were investigated.

MATERIALS AND METHODS

Male Sprague-Dawley albino rats weighing about 200 gm. were injected either with cortisone (50 mg./kg. body weight) dissolved in isotonic saline or with saline alone. The animals were sacrificed three hours after injection and the livers were removed and used for the determination of tryptophan pyrrolase activity (5) and the preparation of the "pH 5 enzyme" fraction by the method of Keller and Zamecnik (9). The amino acid activating enzymes of the "pH 5 enzyme" fraction were determined by measuring the catalysis of exchange of P^{32} between inorganic pyrophosphate and ATP in the presence of no added amino acids, added leucine, valine, methionine, and tryptophan. Leucine has been widely studied in this system and thus served as a control, methionine and valine have been implicated as probable tryptophan pyrrolase constituents (5, 10), and tryptophan is the substrate of the enzyme in question. The exchanges were determined by the method of DeMoss and Novelli (11) and the results were calculated as μmoles exchange/mg. "pH 5 enzyme" protein/hour. Tryptophan pyrrolase activity was calculated as μmoles of kynurenine formed per hour per mg. nondialyzable nitrogen (5).

RESULTS AND DISCUSSION

The results shown in Table 1 are for the cortisone-injected animals and are expressed as the percent of the corresponding control. In all experiments where studied the rat liver tryptophan pyrrrolase activty was stimulated by cortisone injection, and analysis of the absolute values showed that the differences were significant at the 1% level. Administration of the hormone did not significantly alter the rate of P^{32} exchange in the presence of no added amino acids, added leucine, valine, or methionine. The small differences between cortisone-injected and control rats were not significant for any of these even at the 50% level. To obtain a measure of the total rather than specific exchange activities, the results were also calculated as μmoles exchange/gm. rat liver/hour. Analysis of these values again showed small differences between control and cortisone-injected animals, none of which were significant at the 50% level. Tryptophan-dependent exchange was so low that valid comparisons could not be made. Thus, under the condi-

tions used, increased tryptophan pyrrolase activity could not be correlated with an increase in the activity of certain amino acid activating enzymes. This evidence, taken in conjunction with other recent studies (1, 12), renders later steps in protein synthesis or the removal of protein from ribosomes as more likely sites of cortisone action.

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INCORPORATION OF GLUCOSE CARBON INTO PROTEIN OF THE ISLETS OF LANGERHANS FROM TOADFISH (*OPSANUS TAU*)

ABSTRACT

Isolated islets of toadfish have been incubated for periods up to 48 hours at 20° C, and glucose uptake, lactate production, glucose oxidation to CO_2, and incorporation of glucose carbon into protein have been studied. Glucose carbon was incorporated into an acid ethanol extractable protein fraction exhibiting an Rf similar to beef and fish (bonito and tuna) insulin in the system described, as well as biological insulin activity.

Islet tissue in many teleost fishes is not scattered within the exocrine pancreas as in mammals, but forms one or more distinct organs, the Brockmann bodies (1) or principal islets (2). The possibility that islet metabolism might be profitably studied in teleost fishes has occurred to many. Specific attempts have been made by Maske (3) in the flounder, and particularly by Lazarow and his collaborators (4, 5, 6) in the toadfish. Since the toadfish (*Opsanus tau*) is indigenous to the Eastern Seaboard of the United States, is available from May to December, and is of a convenient size, it has been selected for the studies to be reported here.

In preliminary experiments carried out with Lazarow and Ball (7), it was established that toadfish islets contain approximately 140 units of insulin per gram weight, as measured in homogenates with the rat epididymal adipose tissue technique (8). This insulin content agrees remarkably well with the estimates reported for tuna, bonito, cod, mackerel and flounder, and exceeds by a factor of approximately 100 the average insulin content of mammalian pancreas. The present studies were undertaken in order to attempt the simultaneous exploration of metabolic activity and of insulin synthesis in this tissue.

Received March 28, 1961.

METHODS

Toadfish were obtained from the Marine Biological Laboratories, Woods Hole, Mass. They were transported to Boston in cooled containers, and stored in continuously circulated sea water at the American Lobster Company, Boston,[1] remaining in excellent condition for periods as long as several months. On experimental days they were brought to the laboratory in aerated sea water and killed by stunning just prior to use. The largest islet of each fish was rapidly dissected *in situ* and placed in ice-cold buffer. The wet weight of each islet varied between 2 and 6 mg. After harvesting 20 to 60 islets from the same number of fish, they were randomly distributed into groups of 5 to 15 islets, weighed and placed into incubation flasks containing 0.75 ml. Krebs-Ringer bicarbonate buffer or complete Earle's medium (9). Preliminary studies had established that bicarbonate buffer was preferable to phosphate buffer and that tissue survival was not improved by increasing the osmolarity of the bicarbonate buffer. Incubation was carried out for 24 or 48 hours at 20° C in a Dubnoff metabolic shaking incubator. The islets were transferred to new flasks containing sterile medium every twelve hours. The presence or absence of streptomycin (5 mg. %) and penicillin (5 mg. %) did not alter the results obtained. Glucose randomly labeled with C^{14} was obtained from New England Nuclear Corporation and was chromatographically pure. It was used at the concentration of 8 mM and each flask contained 2 to 20 microcuries of radioactivity.

The incubation medium for each 12-hour period was assayed for CO_2, glucose and lactic acid. Incubation flasks containing the same medium, but incubated without islets, served as controls. At the end of 24 or 48 hours the islets of each flask were homogenized in 10% trichloroacetic acid, the precipitate washed 5 times with trichloroacetic acid, heated to 90° C for 15 minutes, and further treated in one of three ways: (a) washed with ethyl ether and plated for counting; (b) extracted with acidified ethanol according to Grodsky (10); the extract was dialysed against four changes of distilled water (using Visking 24/32 tubing), lyophilized, redissolved in acid alcohol according to Jephcott (11), and plated for counting; (c) the redissolved lyophilized extract was subjected to ascending paper chromatography according to Fenton (12), using the organic phase of secondary butanol, acetic acid, water (100:1:99) as solvent, and bromocresol green as staining agent. The amount of protein present was estimated by elution with alkaline ethanol and colorimetry, the radioactivity by scanning of the chromatogram, the total amount of radioactivity applied to each strip being known.

RESULTS AND DISCUSSION

A complete representative experiment (out of nine) using 30 islets is shown in Table 1. Prolonged survival of incubated islets occurs over a period of 48 hours, as measured

TABLE 1. METABOLISM OF GLUCOSE-U-C^{14} (8m*M*, 20 MICROCURIES PER FLASK) BY ISOLATED ISLETS OF TOADFISH (*Opsanus tau*)

Each flask contained 15 islets (approximately 75 mg. wet weight). All values are expressed as either c.p.m. or μM carbon per gram wet weight of islet tissue.

Incubation period (hours)		0–12	12–24	24–36	36–48
Glucose disappearance	(a)	140	132	120	156
μM carbon	(b)	112	124	—	—
Lactate production	(a)	32	32	32	28
μM carbon	(b)	35	28	—	—
Glucose to CO_2	(a)	12.6	20.4	31.3	16.1
μM carbon	(b)	14.6	28.8	—	—
		24 hrs.		*48 hrs*	
TCA precipitate c.p.m.	(b)	1.8×10^6		(a) 4.16×10^6	
μM carbon	(b)	7.7		(a) 17.8	
Acid ethanol extract c.p.m.	(b)	1.05×10^6		(a) 2.47×10^6	
μM carbon	(b)	4.5		(a) 10.6	

[1] The authors are greatly indebted to the American Lobster Company for allowing them access to their storage facilities.

by glucose utilization, oxidation to CO_2, formation of lactate, and incorporation of glucose carbon into islet protein. Approximately one half of the counts incorporated into TCA precipitable protein was extractable with acidified ethanol. Four fractions were regularly obtained upon chromatography of the extract (Fig. 1, B); a fraction remaining at the origin; 2 fractions with Rf similar to crystalline beef insulin (Fig. 1, A); and a

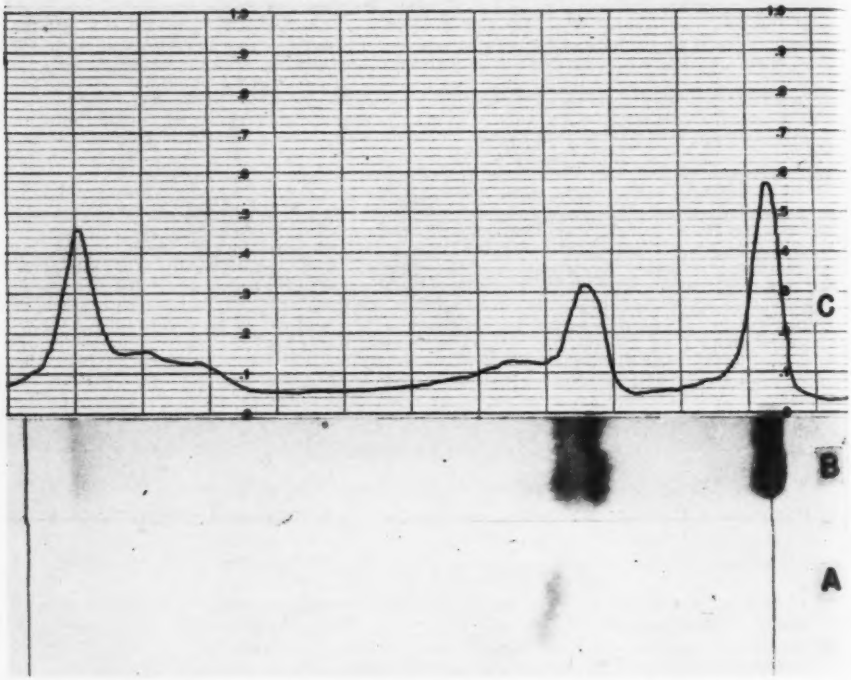

FIG. 1. Chromatography of acid ethanol extract of TCA precipitate obtained after 48 hours of incubation (from experiment shown in Table 1). A. Beef insulin standard (20 μg). B. $\frac{1}{4}$ of extract obtained from 71 mg. islets. C. Radioactivity tracing of extract, set at 5,000 c.p.m.

fraction moving with the solvent front. Radioactivity was found in all four fractions (Fig. 1, C), about 20% of the total counts being present in the "insulin" fractions, resulting in a specific activity of 6.1×10^4 cpm per mg. protein (corresponding to 4.4×10^4 disintegrations p.m.). Two dimensional chromatography in butanol-formic acid-water (4:1:5) did not alter this specific activity. The four fractions obtained were eluted with 1/30N HCl and assayed for insulin activity with both the adipose tissue (8) and the diaphragm techniques (13). The "insulin" fractions exhibited insulin activity, whereas fractions 1 and 4 did not. There is evidence, therefore, that these fractions consist at least in part of insulin. Presence of two insulin fractions in fish has been shown in bonito (14) and flounder (15). The observation that glucose was incorporated into a protein fraction with the chromatographic behavior and biological activity of insulin suggests that the preparation may prove useful in more extensive studies of the factors concerned with the synthesis, storage and release of insulin in islet tissue.

Acknowledgments

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CHANGE OF PUBLISHERS

Following December 31, 1961, The Endocrine Society Incorporated will assume publication of ENDOCRINOLOGY and The Journal of CLINICAL ENDOCRINOLOGY and METABOLISM. All matters and correspondence relating to subscriptions, back volumes, reprints, and advertising may be addressed to the Executive Secretary, The Endocrine Society, Inc., 1200 North Walker Street, Oklahoma City 3, Oklahoma, following December 31, 1961.

THE ENDOCRINE SOCIETY

1962 AWARDS

Fred Conrad Koch Award

In 1957 a substantial legacy was bequeathed to the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957, Dr. I. L. Chaikoff in 1958, Dr. Wilbur W. Swingle in 1959, Dr. Emil Witschi in 1960 and Dr. Lawson Wilkins in 1961. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator who has not attained his 36th birthday by June 1st of the year the award is presented, in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer, 1959 Dr. Gordon L. Farrell, 1960 Dr. Don H. Nelson and 1961 Dr. Ernst Knobil. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $7,500 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Upjohn Awards

The Council of The Endocrine Society has approved a change in status of the Upjohn Scholar to that of The Upjohn Awards.

There will be two awards in the amount of $1,250 each, one for the best article in format and content printed in ENDOCRINOLOGY and one in THE JOURNAL OF CLINICAL ENDOCRINOLOGY AND METABOLISM during the preceding year.

Awardees will be chosen by the Awards Committee at its regular meeting in November of each year.

ERRATUM

In the article "Response of the Pineal and Adrenal Glands to Sodium Restriction" by N. M. Panagiotis and G. F. Hungerford appearing in the August issue of ENDOCRINOLOGY (vol. 69 p. 217, 1961) the legends appearing under figures 4 and 5 are reversed.

INTERNATIONAL CONGRESS ON HORMONAL STEROIDS

MILANO, ITALY—14TH–19TH—MAY 1962

The International Congress on Hormonal Steroids will be held in Milano, Italy, from May 14 through 19, 1962; Dr. Gregory Pincus will be the Honorary President and Prof. Emilio Trabucchi, the General Chairman.

The Meeting will consist of 6 Symposia, of 8 Round Table Discussions and of groups of shorter (ten minutes) communicated papers. The topics of the Symposia will be:

1) new steroids with hormonal-like activities
2) pathways of synthesis and metabolism of hormonal steroids
3) control of synthesis and release of steroid hormones
4) mechanism of steroid action
5) substances which affect the synthesis and action of steroid hormones
6) non-hormonal activities of steroids

The following Round Table Discussion are planned:

1) recent advances in steroid chemistry
2) new anabolic steroids
3) progesterone and progestational compounds
4) action of hormonal steroids on anterior pituitary function
5) free and conjugated steroid hormones and metabolites in blood
6) the adrenogenital syndrome
7) action of hormonal steroids and their derivatives on experimental tumors and tissue regeneration
8) action of hormonal steroids on the central nervous system and behaviour

Speakers of the Symposia and of the Round Table Discussions are invited by the Congress. Communicated papers may be submitted by investigators interested in the field. Forms for Registration, along with forms for the submission of abstracts of short communications may be obtained by writing to the Congress Secretaries: prof. L. Martini and prof. A. Pecile, Department of Pharmacology of the University, 21 Via A.del Sarto, Milano, Italy. The final date for the submission of such abstracts is January 31, 1962. The official languages of the Congress are English, French and Italian. Honorary President: Dr. Gregory Pincus, Research Director, Worcester Foundation, Shrewsbury, Mass., U. S. A.

ANNOUNCEMENT

OF THE

FIFTH INTERNATIONAL PIGMENT CELL CONFERENCE

October 11th–14th, 1961

NEW YORK CITY

Sponsored by

THE NEW YORK ACADEMY OF SCIENCES

Seventy papers covering recent developments in the field of pigment cell biology and related disciplines will be presented during a four-day conference.

Fundamental studies on cancer and other cellular processes will be brought together with related presentations of a clinical nature.

The general areas encompassed by this conference include the natural history and treatment of melanoma, cytology and pathology of benign and malignant pigment cells, hormone and genetic aspects, influence of radiation, transmissible factors, biochemistry and enzymology of melanocytes, and the chemistry of pigmentation processes.

PROGRAM SYNOPSIS

Wednesday, October 11th, 1961

(A) ABNORMAL AND MALIGNANT MELANOCYTES IN HUMANS: THEIR OCCURRENCE, RECOGNITION AND SIGNIFICANCE.
Session Chairman: Ronald Raven, Royal Cancer Hospital, London, England.

(B) THE MALIGNANT HUMAN MELANOCYTE: ITS CONSEQUENCE AND CONTROL PROBLEMS.
Session Chairman: George T. Pack, Memorial Hospital, New York, N. Y.

Panel Discussion

TREATMENT OF MELANOMA

Chairman: Gordon McNeer, Memorial Hospital, N. Y.

Thursday, October 12, 1961

(A) NATURE, RESPONSE, AND INDUCTION OF MELANOCYTES.
Session Chairman: R. D. Passey, Chester Beatty Research Institute, London, England.

(B) NATURE, RESPONSE, AND INDUCTION OF MELANOCYTES (*Cont.*).
Session Chairman: Stephen Rothman, University of Chicago, Argonne Cancer Research Hospital, Chicago, Ill.

(C) Symposium—MELANIN GRANULES, THEIR NATURE AND ORIGIN.
Session Chairman: M. J. Kopac, Graduate School of Arts and Science, New York University, New York, N. Y.

Friday, October 13th, 1961

(A) HORMONES IN PIGMENT CELL MECHANISMS.
Session Chairman: Aaron B. Lerner, Yale University School of Medicine, New Haven, Conn.

(B) Melanocyte Genetics—Classical and Neoclassical.
Session Chairman: R. E. Billingham, Wistar Institute, Philadelphia, Pa.

Saturday, October 14th, 1961

(A) Molecular and Macromolecular Aspects of Melanogenesis.
Session Chairman: G. A. Swan, University of Durham, King's College, New Castle-upon-Tyne, England.

(B) Molecular and Macromolecular Aspects of Melanogenesis (*Cont.*).
Session Chairman: C. R. Dawson, College of Physicians and Surgeons, New York, N. Y.

This conference is made possible by support from the funds of The New York Academy of Sciences and a grant from the Damon Runyon Fund, New York, N. Y.

WEDNESDAY SESSIONS WILL BE HELD AT
Memorial Sloan-Kettering Cancer Center
410 East 68th Street, New York 21, N. Y.
ALL OTHER SESSIONS WILL BE HELD AT
The Henry Hudson Hotel
353 West 57th Street, New York, N. Y.

Vernon Riley
Sloan-Kettering Institute for Cancer Research, Rye, N. Y.
Joseph G. Fortner
Memorial Sloan-Kettering Cancer Center, New York, N. Y.
Conference Cochairmen

CONGRESS OF ENDOCRINOLOGY

The Second Asia & Oceania Congress of Endocrinology will be held at the University of Sydney, New South Wales, Australia, from 28th May to 2nd June, 1963.

P. J. Claringbold,
Congress Secretary

AMERICAN PSYCHOSOMATIC SOCIETY
265 Nassau Road, Roosevelt, New York

The 19th Annual Meeting of the American Psychosomatic Society will be held at The Sheraton Hotel in Rochester, New York, on Friday, Saturday, and Sunday, March 30, 31, and April 1, 1962.

The Program Committee will welcome abstracts of original work to be presented at the Meeting either by members or non-members of the Society. Abstracts should be not more than two typewritten pages, and should be submitted in eleven copies. Deadline for submission is December 1, 1961.

Abstracts should be addressed to the Chairman, 265 Nassau Road, Roosevelt, New York.

Stewart Wolf, M.D.
Chairman, Program Committee